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CHELATION PHENOMENA

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* This series of papers is the result of a conference on *Chelation Phenomena* held by The New York Academy of Sciences on December 7 and 8, 1959.

Part III. The Medical Applications of Chelating Agents

Part IV. The Industrial Applications of Chelating Agents

INTRODUCTION TO THE MONOGRAPH

Jerome F. Fredrick
Research Laboratories, Dodge Chemical Company (Boston, Mass.), New York, N. Y.

"The pursuit of knowledge can never be
anything but a leap in the dark. . . ."
—SAMUEL BUTLER

It is hoped by the contributors to this monograph that its publication will cause "the dark" to be at least partially illuminated. Chelation, that most important and yet most elusive of physical phenomena, was first mapped, so to speak, by Martell and Calvin.[1] With increasing knowledge of chelation has come an appreciation of its importance and an awareness of its universality.

The bridging of the gap between the classic inorganic and organic worlds, far from being of academic interest alone, assumes biological vitality via the chelates, perhaps even to the point where those ancient hemo compounds of Granick's[2] were indeed the first catalysts of life. Certainly, chelates such as chlorophyll, hemoglobin, vitamins, hormones, and enzymes are the propagators and maintainers of present-day life. Besides their essential biological role, chelates and the process of chelation are of increasing interest in hematology, physiology, cancer therapy, decontamination of fission products, and almost every aspect of modern medicine. Their possible auxin relations are of extreme importance in agriculture and the botanical sciences. Chelation opens up new vistas for chemical syntheses, facilitates chemical and food processing and, in general, makes infinitely more comfortable the life it has possibly originated and most certainly maintained.

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Part I. The Chemistry of Chelation

CHELATION: STABILITY AND SELECTIVITY

Arthur E. Martell
Department of Chemistry, Clark University, Worcester, Mass.

This monograph on chelation phenomena embraces a unique variety of subject matter as well as background and interest of the authors represented: among them are chemists interested in chelation as a chemical phenomenon, industrial chemists interested in putting chelates to good use, and other investigators working in the life sciences concerned with the role of chelates in the body and in biological systems generally. Jerome Fredrick is to be congratulated for putting together a program so remarkably varied and yet well integrated as to cover the broad area of research and applications in this ever-expanding field of metal chelate compounds.

I like to look upon the conference on which this monograph is based as an entropy process. Each scientist contributing to it has worked along lines of specific interest to him, and produced results that usually have varied considerably from those of other workers. The bringing together of so many specialists with such varieties of information results in a net loss of entropy. In this monograph, the decrease of entropy is especially great. Thus the resulting spontaneous processes, involving the exchange of information and the subsequent applications of this new information in the laboratories of the participants, are characterized by an especially large entropy increase. It is readily seen, therefore, that this monograph in particular should be highly productive of results, as are all processes involving large increases of entropy.

These entropy changes are analogous to the chelation phenomenon. To achieve the highest possible stability, one designs a chelating agent, or ligand, with the lowest possible entropy. Therefore, when this agent combines with a metal ion, also of low entropy, the entropy increase is particularly great, resulting in a highly spontaneous reaction. It is only natural, therefore, to look a little more closely at this chelation reaction to find out in a general way what information has been gained from it and what one may conclude about its future directions.

One of the outstanding advances in coordination chemistry was the determination, about fifteen years ago, of the stabilities of the alkaline earth complexes of aminopolycarboxylic acids, of which ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) were and still are the outstanding examples. Until that time it was thought impossible to effect reactions of the type indicated in FIGURE 1. The explanation of the high stability of such complexes of the more basic metal ions gradually became obvious from the determination of the stability constants of numerous metal chelate compounds, and it is based on concepts of the so-called chelate effect and an understanding of the general nature of the reactions involving the combination of positive and negative ions.

Perhaps the most important factor in obtaining high stability in metal che-

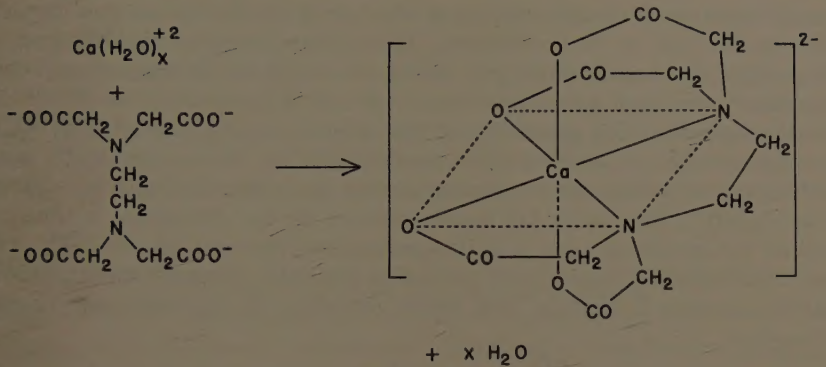

FIGURE 1.

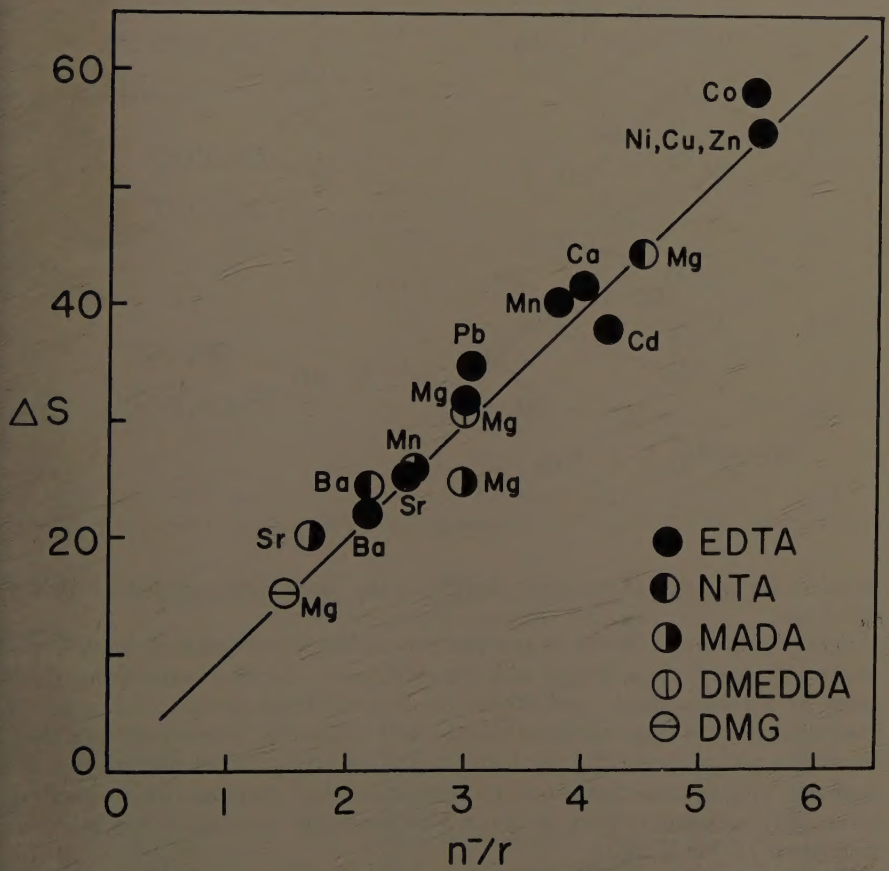

FIGURE 2. Entropy increases for the combination of various ligands with divalent metal ions; n^-, number of acetate groups of the ligand that combine with the metal ion; r, crystal radius in Ångström units; ligands are EDTA (ethylenediaminetetraacetic acid), NTA (nitrilotriacetic acid), MADA (methylaminediacetic acid), DMEDDA (N, N′-dimethylethylenediaminediacetic acid), and DMG (dimethylglycine).

lates of this type is the dissociation of water from the highly solvated positive and negative ions as they combine. This process involves neutralization of the positive and negative charges, with a release of solvated water molecules from both cation and anion, resulting in an entropy increase that drives the reaction forward. The generality of this effect is seen in FIGURE 2, in which the entropy changes involving the combination of various divalent metals and aminocarboxylic acids are plotted against the number of negative charges of the ligand.[1] The factor 1/r merely corrects for the differences in the ionic radii of the metals to allow a better comparison between ligands. The fact that experimentally determined quantities generally fall near the line shows that the increase in entropy and, hence, stability, is approximately propor-

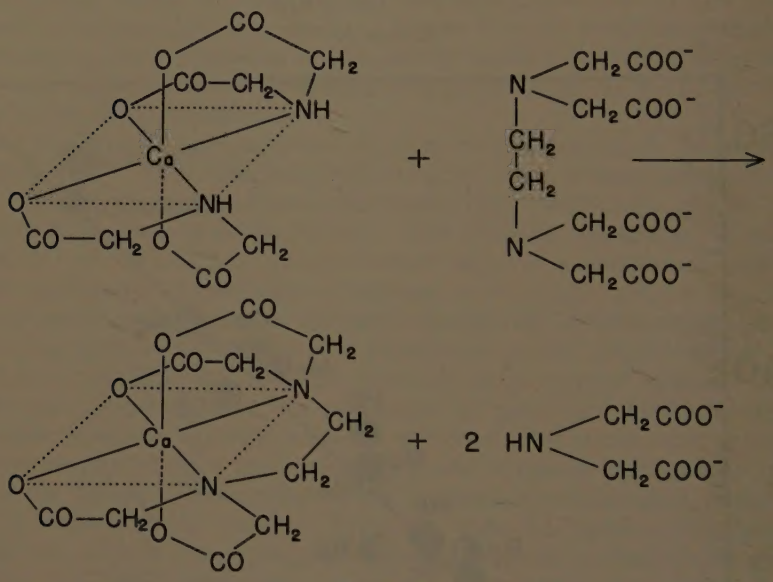

FIGURE 3.

tional to the number of negative charges of the ligand that associate with the metal ion.

Another important factor in the solution stabilities of metal chelates is the number of chelate rings formed with the metal ion. An interesting comparison made by Schwarzenbach[2] and others involves the displacement of 2 moles of iminodiacetic acid from its metal chelate by 1 mole of ethylenediaminetetracetic acid, a process involving the formation of an additional chelate ring in accordance with the reaction shown in FIGURE 3. This reaction, which involves an entropy increase, is seen in TABLE 1 to result in a stability increment of more than 2.0 log K units.

The high stabilities of EDTA chelates, therefore, are due in large part to relatively large entropy increases, as may be seen from the data in TABLE 2.[1] In the case of the alkaline earth ions, the small negative values of ΔH show

that the increase in entropy is the only important driving force in the formation of the aqueous chelate compounds.

Along with this development of a theoretical explanation of the high stabilities of these alkaline earth chelates there remains the problem of increased selectivity. The creation of highly selective chelating agents always has interested investigators who use them, whether in the analytic determination of metals, the separation of metal ions for preparative purposes, or the study of

TABLE 1

RELATIVE CHELATING TENDENCIES OF TWO MOLES OF IMINODIACETATE AND ONE MOLE OF ETHYLENEDIAMINETETRAACETATE

M(II)	Log K_{IMDA}	ΔLog K_{IMDA}	Log K_{EDTA}	ΔLog K_{EDTA}	Log K_{EDTA} − Log K_{IMDA}
Ca	5.4		10.6		5.2
		4.6		5.9	
Cd	10.0		16.5		6.5
		0.1		−2.5	
Fe	10.1		14.0		3.9
		2.1		2.0	
Zn	12.2		16.0		3.8
		0.1		0.2	
Co	12.3		16.2		3.9
		2.3		2.0	
Ni	14.6		18.2		3.6
		1.5		0.3	
Cu	16.1		18.5		2.4

TABLE 2

THERMODYNAMIC CHANGES ASSOCIATED WITH THE FORMATION OF EDTA CHELATES IN AQUEOUS SOLUTION

Metal ion	$-\Delta F°$ (kcal./mole)	$-\Delta H°$ (kcal./mole)	$\Delta S°$ (cal./° C.)
Ca(II)	15.0	3	42
Sr(II)	11.9	4	26
Ba(II)	10.5	4	22
Mn(II)	17.2	5.2	41
Co(II)	21.4	4.1	58
Ni(II)	24.0	7.6	55
Cu(II)	24.4	8.2	55
Zn(II)	20.9	4.5	55
Cd(II)	20.5	9.1	38
Pb(II)	23.6	13.1	35

specific effects in biological systems. From the data in TABLE 1, however, it is seen that increasing the stability through increasing the number of chelate rings in general does not increase selectivity between metals. The stability constants listed show that EDTA does not differentiate between metal ions

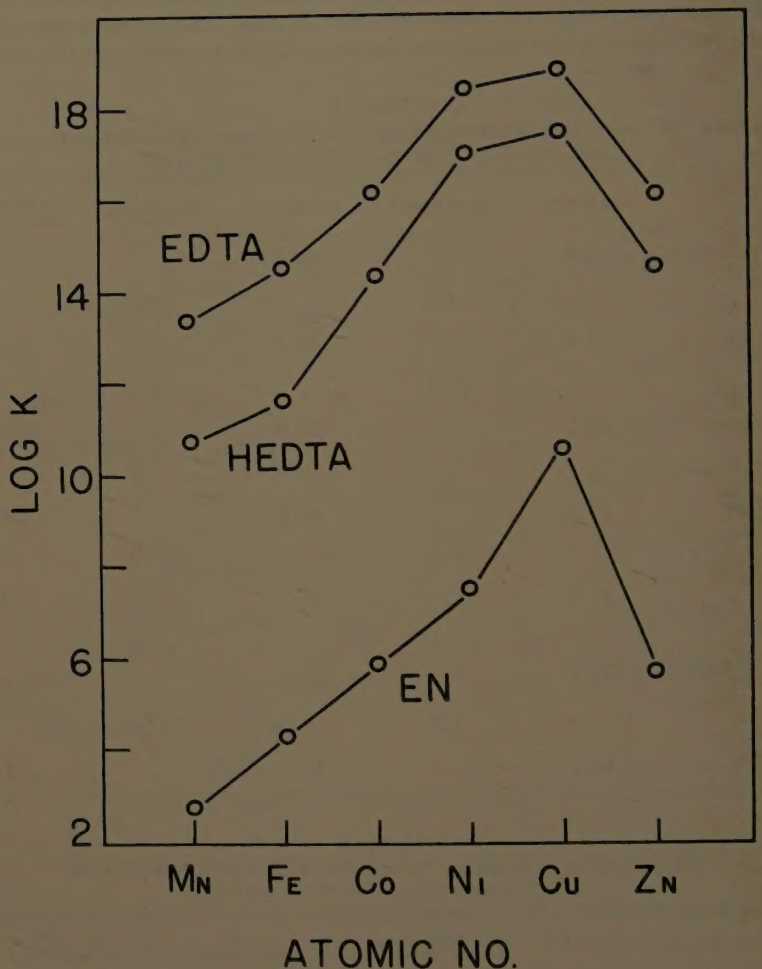

FIGURE 4. Chelating tendencies of ethylenediaminetetraacetic acid (EDTA), N-hydroxyethylethylenediaminetriacetic acid (HEDTA), and ethylenediamine (EN), as a function of metal ions of the first transition series arranged in order of increasing atomic numbers.

any more than does iminodiacetic acid. This is true also of many other chelating agents that seem to have considerably less "chelating power." The chelate effect and other special properties that obtain in the complex reactions of EDTA, therefore, are such as to increase affinity for all metal ions. Thus, while EDTA and similar compounds are characterized by very high stabilities

of their chelates with all metal ions, they do not have the properties that would make them very selective.

A clue to the requirements for selectivity may be seen in FIGURE 4, which shows the stabilities of the transition metal chelates of EDTA and ethylenedi-

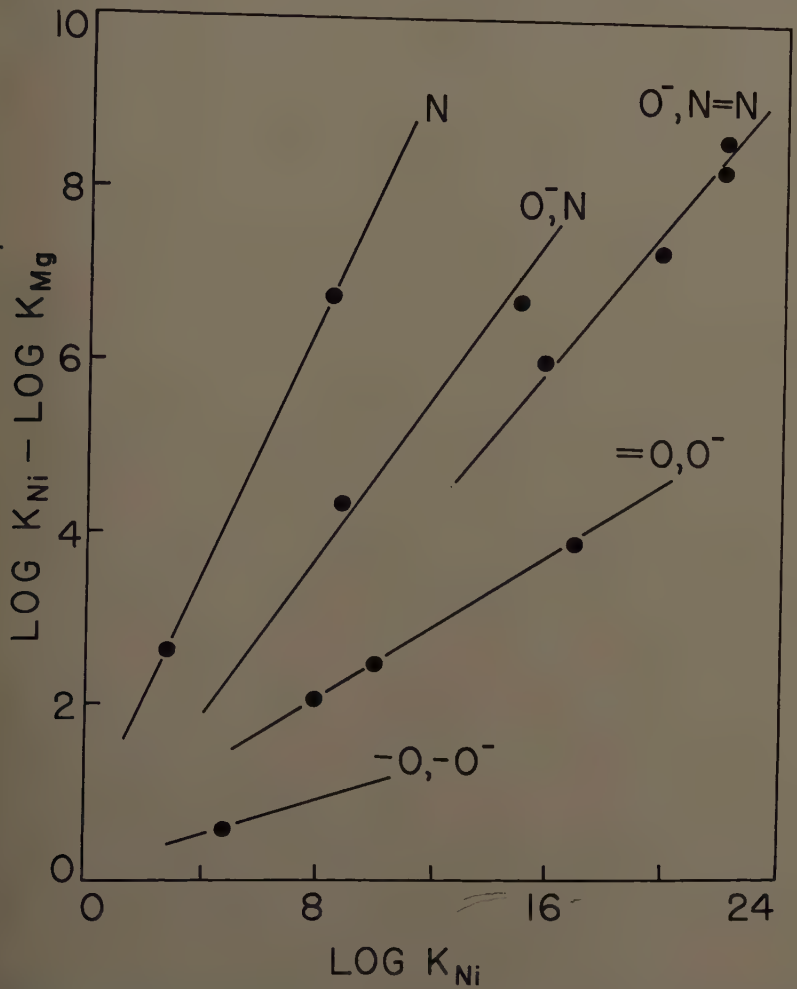

FIGURE 5. Relative stabilities of the Ni(II) and Mg(II) chelates as functions of the nature of the coordinating groups in the ligand.

amine.[3] It is seen that ethylenediamine, although a poorer chelating agent, is nevertheless much more selective than is EDTA and the analogous compound, hydroxyethylethylenediaminetriacetic acid (HEDTA). The fact that ethylenediamine has only nitrogen donors, whereas EDTA has both nitrogen and oxygen groups for coordination, is significant in the differentiation of the transition metals, as well as of the transition metals and the alkaline earth ions.

Increasing the number of chelate rings of the ligand by substituting acetate groups on ethylenediamine to give EDTA or HEDTA thus greatly increases stabilities of the metal chelates formed, but decreases selectivity.

The role of the nature of the donor atom in promoting selectivity may be seen from the data plotted in FIGURE 5, which gives the differences between the stabilities of the nickel and magnesium chelates formed from bidentate chelating agents having various types of coordinating groups.[4] The slopes of the lines, which are indicative of selectivity, show that the greatest differences occur when the ligand contains only nitrogen donors and is least when the ligand contains only oxygen atoms as complexing groups.

TABLE 3
Ni(II) AND Pb(II) CHELATING TENDENCIES OF LIGANDS CONTAINING BASIC SULFUR GROUPS

Ligand	Log K_f		K_{Pb}/K_{Ni}
	Ni(II)	Pb(II)	
$CH_3—S—CH_2CH_2—N(CH_2COO^-)_2$	10.00	9.12	0.13
$^-OOCCH_2—N(CH_2COO^-)_2$	11.53	11.39	0.72
$^-SCH_2—CH_2—N(CH_2COO^-)_2$	13.75	17.03	1950

The importance of changing the nature of the donor group to achieve selectivity is strikingly illustrated in TABLE 3, which shows the remarkable difference in stabilities of nickel and lead chelates brought about by changing an aliphatic carboxyl group to a thiol group.[5] The differences are relatively small with NTA and with the thioether, but the stabilities of the nickel and lead chelates of the corresponding thio compound differ by a factor of nearly 2000.

Another way of achieving selectivity is in the geometry of the ligand molecule, which may be designed to take advantage of the relatively small differences between the ionic radii of the metal ions. An excellent example of the achievement of selectivity through steric effects may be seen in FIGURE 6, which indicates the structure of the metal chelate of 2-methyl-8-hydroxyquinoline.[6] This compound does not form a precipitate with the Al(III) ion, which is only slightly smaller than other tripositive ions such as those of Fe(III), Cr(III), Ga(III), In(II), and Tl(III). All these ions, including those of Al(III), are

readily precipitated by 8-hydroxyquinoline itself. It seems, therefore, that the methyl groups inhibit the formation of the Al(III) chelate, in which they would be somewhat closer to the oxygen and nitrogen atoms at the points indicated by solid dots. Achievement of such steric effects requires considerable rigidity in the ligand and can best be obtained in aromatic compounds, where the positions of the ring substituents are fixed relatively more firmly than is generally true of the more flexible aliphatic ligands.

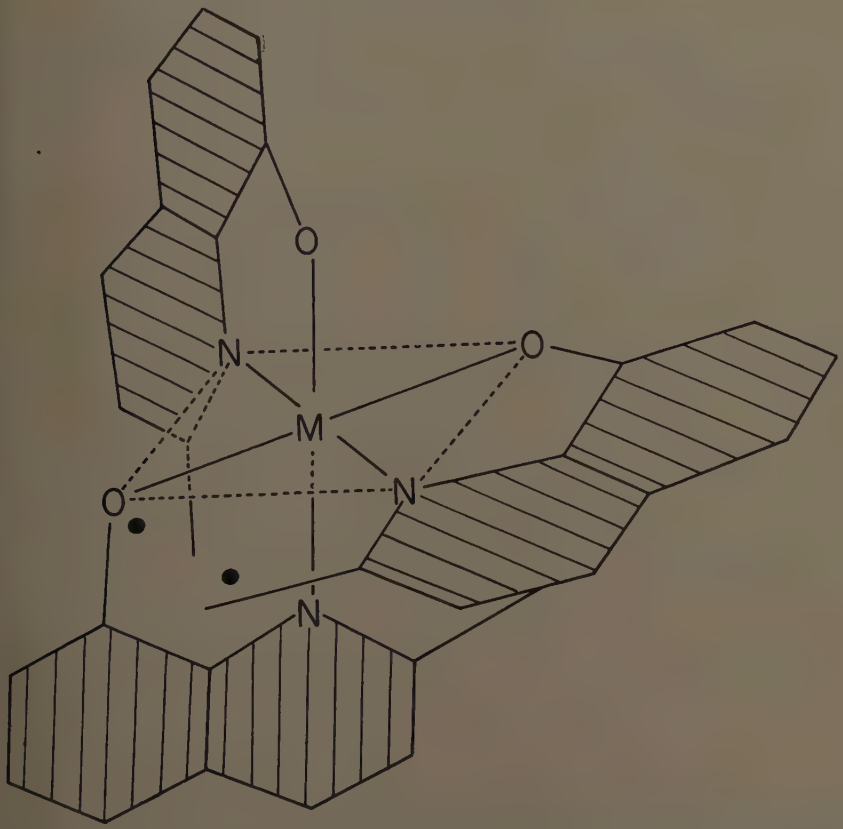

FIGURE 6. Schematic representation of steric hindrance in 2-substituted 8-hydroxyquinoline chelates of tervalent metal ions.

The examples given above are representative of the general behavior shown by chelating ligands and indicate that factors such as negative charge of the ligand and number of chelate rings formed increase the affinity of the ligand for all metal ions. On the other hand, selectivity in chelating agents probably is best achieved by factors that differ most from metal ion to metal ion. Thus it is possible to take advantage of differences in electronegativity of metal ions (that is, differences in tendencies to form covalent bonds) by changing the nature of the donor atom. The less electronegative ligands are most effective

for the less electropositive metals and are least effective for highly electropositive metal ions. Also, the rigid positioning of donor groups in a ligand can give selectivity by steric effects, such as those arising from mutual repulsions of ligand groups or from changing the fit of the ligand about a metal ion.

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INDICATIVE PROPERTIES OF A FERRIC CHELATE

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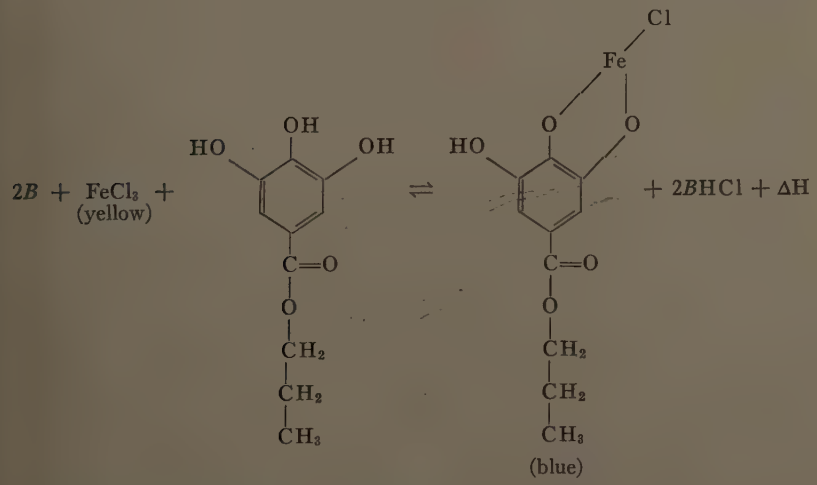
Many Fe(III) complexes and chelates that do not undergo disproportionation or permanent reaction with the solvent show thermochromism and solvochromism. The term thermochromic is used to describe a visible color change in a given solution due to a temperature differential. The term solvochromic indicates a visible color change as a result of a change in composition of a solution at constant temperature. To illustrate, a solution of *p*-cresol, ferric chloride, and pyridine may be yellow, orange-brown, green, blue, or violet, depending on the relative concentrations of the constituents, solvent, *p*H or its equivalent in nonaqueous media and temperature. Whereas solutions of ferric chloride, *n*-propyl gallate, and *o*-chloroaniline are blue throughout the liquid temperature range of water, methanol, and dimethylformamide, the same system is yellow throughout the same range in diethyl ether, acetone, and methyl acetate. A similar behavior has been observed between Fe(III) and some other phenols, enols, oximes, hydroxamic acids, and amidoximes.[1]

In many instances color changes occurred over such narrow ranges of temperature and composition that the particular system seemed to possess useful properties as an indicator. The chelate formed between ferric chloride and *n*-propyl gallate was chosen for such an investigation as it seemed stabler to temperature than some of the other complexes. The equilibrium between the parent constituents forming the chelate may be represented as follows:



(1)

where *B* is a base such as *o*-chloroaniline.

It is readily appreciated that the above formulation is oversimplified, particularly in view of the range of solvents, concentrations, and temperatures at which the system was studied.

The obvious possibilities of the chosen system as a *p*H and redox indicator in aqueous solutions were only briefly investigated. It was shown that water is readily detectable in alcoholic solutions to the extent of a few per cent.[2] The displacement of the equilibrium toward a greater chelate concentration is due to the greater basicity of water over alcohols. Our major interest lay in studying the behavior of the chelate in a variety of nonaqueous media.

For a rapid survey of the effect of solvent and temperature two solutions were prepared.[3] The first, indicator A, was a dilute solution of ferric chloride and *n*-propyl gallate in glacial acetic acid. The second, indicator B, consisted of a dilute solution of indicator A and *o*-chloroaniline in bromobenzene. Test A consisted of adding 1 drop of indicator A and 1 drop of *o*-chloroaniline to 1 ml. of a liquid. Test B consisted of adding 3 drops of liquid to 1 ml. of indicator B. In this manner, the effects of wide differences in the concentration of the substance under test could be observed. In each instance the critical thermochromic temperature (CTT) was noted. If the added components yielded a yellow solution, it was cooled until it became blue. Minus 10° C. was the lowest temperature in this series of experiments. If the resulting solution was blue at room temperature, it was heated until it became yellow and then allowed to recool slowly so as to more accurately observe the CTT. Since the concentration of the component under test plays some role, and because the amount of the component was only controlled to perhaps 10 per cent, the CTT measurements obtained have no absolute significance. Each compound was assigned a group designation which, in turn, represented a range of CTT. These designations are given in TABLE 1.

The results in TABLE 2 list types of compounds in order of increasing ability to function as inhibitors of chelation. Many substances destroyed the indicator by irreversible oxidation. Some of the known compounds that showed this behavior were: *t*-butyl perbenzoate, cumene hydroperoxide, *t*-butyl hypochlorite, and *N*-bromosuccinimide. Others of unknown composition were old shelf samples of olefins, ethers, and aldehydes. However, when the latter were freed of such compounds as peroxides and hydroperoxides, they behaved as classified in TABLE 2.

The reasons for the sulfonic and phosphonic acid inhibitory properties seemed obvious enough, as did the promotional effects of amines. However, the strong promotional effects of bulk phenols and amides, the weak promotional effects of bulk alcohol, and the inhibitory effects of such compounds as ethers, aldehydes, and ketones posed some puzzling problems. An examination of some of the exceptions to the classifications given in TABLE 2 is helpful toward an understanding of these effects. For example, consider the change in classification of ethyl acetate with stepwise chlorination:

ethyl acetate	I_m^s	I_d^w
ethyl chloroacetate	I_m^s	N_d
ethyl dichloroacetate	I_m^w	N_d
ethyl trichloroacetate	N_m	N_d

TABLE 1

KEY TO THERMOCHROMIC DESIGNATIONS

	−10° C.	20° C.	40° C.	100° C.	125° C.
Blue (?) ⇌		Blue ⇌ yellow	Blue ⇌ yellow	Blue ⇌ yellow	Blue ⇌ yellow (?)
Test / Yellow					
A	I_m^s	I_m^w	N_m	P_m^w	P_m^s
B	I_d^s	I_d^w	N_d	P_d^w	P_d^s

Indicator B had a critical thermochromic temperature of 34° ± 2° C. If an added compound elevated the critical thermochromic temperature above 40° C. in either test, it was designated as a promoter of chelation, P. A depression of the critical thermochromic temperature below 20° C. categorized a compound as an inhibitor, I. Any substance which gave a value between 20° and 40° C. was termed a neutral, N. Because of concentration dependence a few cases of overlap at these artificial boundaries were observed. Superscripts are used to subdivide the promoter and inhibitor classes further. Superscript w (weak) attached to I refers to the fact that the chromic transition was observed between −10° and 20° C. in both tests. Superscript w attached to P refers to a chromic transition in the region of 40° to 100° C. in test A and 40° to 125° C. in test B. Superscript s (strong) means that a chromic transition may occur below −10° C. for an inhibitor, above 100° C. for a promotor in Test A, or above 125° C. in Test B.

TABLE 2

CLASSIFICATION OF TYPES OF ORGANIC COMPOUNDS IN ORDER OF INCREASING ABILITY AS INHIBITORS OF CHELATION

Type of compound	Thermochromic indicator	
	A	B
Aliphatic amine	P_m^s	P_d^s
Aromatic and heterocyclic amines	P_m^s	P_d^w
Phenol	P_m^s	N_d
Aliphatic amide	P_m^s	I_d^s
Alcohol	P_m^w	I_d^w
Carboxylic acid	N_m	N_d
Diaryl ether	N_m	N_d
Hydrocarbon	N_m	N_d
Mercaptan	N_m	N_d
Aromatic nitro compound	I_m^w	N_d
Aryl alkyl ether	I_m^w	N_d
Aromatic nitrile	I_m^w	I_d^w
Aliphatic nitro compound	I_m^s	N_d
Alkyl aryl sulfonate ester	I_m^s	N_d
Aliphatic monoether	I_m^s	I_d^w
Aliphatic nitrile	I_m^s	I_d^w
Aryl alkyl ketone	I_m^s	I_d^w
Ester	I_m^s	I_d^w
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Aliphatic acid anhydride	I_m^s	I_d^s
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Carboxylic acid chloride	I_m^s	I_d^s
Phosphonic acid	I_m^s	I_d^s
Saturated heterocyclic ether	I_m^s	I_d^s
Sulfonic acid	I_m^s	I_d^s
Trialkyl phosphate	I_m^s	I_d^s

Because of the relative electronegativity of Cl to H the basicity of the molecule is decreasing with an increasing number of chlorine atoms. An examination of TABLE 2 shows that arylation decreases the effectiveness of a functional group as an inhibitor.

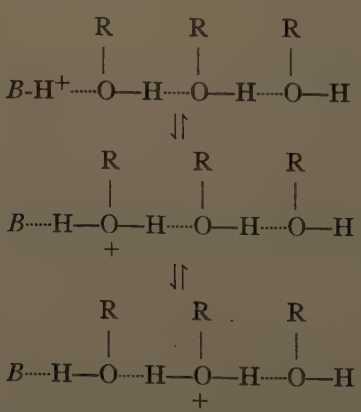
Note the following cases:

(1)	aliphatic polyethers	I_m^s	I_d^s
	dialkyl ethers	I_m^s	I_d^w
	aryl alkyl ethers	I_m^w	N_d
	diaryl ethers	N_m	N_d
(2)	aliphatic nitro compound	l_m^s	N_d
	aromatic nitro compound	l_m^w	N_d
(3)	aliphatic nitrile	l_m^s	l_d^w
	aromatic nitrile	l_m^w	l_d^w
(4)	dialkyl ketone	l_m^s	l_d^s
	aryl alkyl ketone	l_m^s	l_d^w

The parallel decrease of basicity and inhibitory power of chelation in these cases suggests a relative decrease of extent of reaction with an acid. Lemaire and Lucas[4] demonstrated that dibutyl ether, acetone, and ethylene diacetate are at least 10^6 times weaker as proton-accepting bases than *o*-chloroaniline. Hence the strong inhibitors of chelation are functioning as bases toward ferric chloride and are competing against the chelating agent for the coordination sphere of Fe(III).

It is interesting to note at this point that alcohols in dilute bromobenzene solution (test B) are I_d^w for the most part, whereas heterocyclic ethers and dialkyl ethers are I_d^s and I_d^w respectively. Hence, as the degree of hydrogen bonding in the former type of compound decreases with dilution and the population of "isolated" alcohol molecules increases, the behavior is similar to ethers. The behavior of bulk alcohols and phenols as promotors of chelation is probably due to their hydrogen bonded state. In this state a positive charge can be shared throughout the liquid as shown in the following picture:

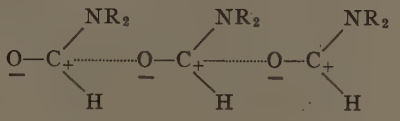
$$A\text{-H} + B \rightleftharpoons A^- + B\text{-H}^+$$



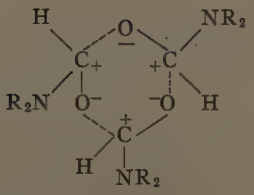
(2)

A similar explanation is offered for the low molecular weight unsubstituted

and *N*-monoalkyl substituted amide derivatives. However, hydrogen bonding is virtually absent in dimethyl formamide. If one assumes strong dipole-dipole interaction to form chains or rings, as pictured below, then the dialkyl amino groups approach aliphatic amines in strength as proton acceptors. The relatively high boiling point of dimethyl formamide in view of its low molecular weight also suggests the presence of strong intermolecular forces.



and/or (3)



Compounds such as *N*-*n*-butyl acetamide, *n*-butyl lactate, diethyl tartrate, and benzonitrile are weak inhibitors regardless of the state of dilution (tests A and B). Dilution of the bulk state, whether hydrogen bonded or dipole-dipole, promotes dissociation to the "isolated" molecule. If the concentration of the "monomeric" state varies little with dilution, and it is the more potent factor than the "polymeric" state in displacing the thermochromic equilibrium, we can understand this buffer effect.

Quantitative Estimation of Basicity

A study of the dependence of the CTT of the chelate in bromobenzene as a function of base strength led to a simple method for the estimation of the pK_b values of aromatic and heterocyclic amines.[5] The solutions, whose CTT values were measured, were prepared by adding 5 drops of indicator A and 5 drops or 150 to 170 mg. of an amine to 7.5 ml. of bromobenzene. In the case of aliphatic amines the solutions were blue from room temperature to the boiling point. The solutions containing the aromatic derivatives became yellow around 125° C., and were then allowed to cool slowly with gentle shaking until the color just became blue to the visual absence of green. These CTT values were reproducible to ±2° C. They are listed in TABLE 3.

Assuming a constant heat of reaction between an acid and a family of bases, one can treat thermochromic equilibria by the van't Hoff equation. The equilibrium under study can be represented by the following operational symbols: where *A*-H is a combination of ferric chloride and *n*-propyl gallate, and *B* is an aromatic or heterocyclic amine. In a low dielectric solvent such as bromobenzene the conjugate base of the acid is assumed to be a hydrogen bonded adduct (*A*-H-*B*). The equilibrium constant, K_b, is given by the conventional expression and is a measure of the strength of *B*.

$$K_b = \frac{(A\text{-H-}B)}{(A\text{-H})(B)} \qquad (4)$$

In the experiments[5] the mole ratios of ferric chloride to *n*-propyl gallate to various bases were 1 to 1.5 to 28–58. Hence the concentration of *B* remains substantially constant, and the equilibrium constant expression reduces to:

$$K'_b = \frac{(A\text{-H-}B)}{(A\text{-H})} \qquad (5)$$

The van't Hoff equation relates the equilibrium constants at the CTT values and 298° K as shown in (6):

$$\log \frac{K\ (298°\ K)}{K\ (CTT)} = \frac{\Delta H}{2.3R}\left[\frac{1}{CTT} - \frac{1}{298}\right]$$

or (6)

$$pK'_b\ (CTT) - pK'_b\ (298°\ K) = \frac{\Delta H}{2.3R}\left[\frac{1}{CTT} - \frac{1}{298}\right]$$

TABLE 3

BASICITY CONSTANTS OF AROMATIC AMINES FROM THERMOCHROMIC MEASUREMENTS

Aromatic amine	CTT(° C.)	pK_b (calculated)	pK_b (literature*)
2,5-Dichloroaniline	5	13.2	
o-Bromoaniline	26	12.2	
o-Chloroaniline	34	11.9	12.0
m-Bromoaniline	79	10.3	
m-Chloroaniline	81	10.2	10.4
Dimethylaniline	98	9.8	9.6
p-Chloroaniline	102	9.7	9.8
p-Bromoaniline	102	9.7	10.0
p-Toluidine	105	9.5	9.7
m-Toluidine	105	9.5	9.3
Aniline	105	9.5	9.3
o-Toluidine	110	9.4	9.5
6-Methylquinoline	113	9.3	

* These pK_b values were calculated from tables of dissociation constants in the *Handbook of Physics and Chemistry*[10] (ed. 34), p. 1560 and from other sources.

At the measured CTT values, the observed color is the same for all bases. Hence, the K'_b values are identical at those temperatures. The van't Hoff equation, therefore, simplifies to:

$$pK'_b\ (298°\ K) = \frac{k_1}{CTT} + k_2 = \frac{3860}{CTT} - 0.7 \qquad (7)$$

The pK'_b (298° K) values given in TABLE 3 were calculated from the above equation. The slope of the line gives a heat of neutralization of 17.7 kcal. This value compares favorably with many of those given by Brown[6] (17.1 to 18.4 kcal.) for the neutralization of methane sulfonic acid by typical heterocyclic amines in the aprotic solvent, nitrobenzene.

Degree of Hydrogen Bonding in Alcohols

Because of the previously observed promotional action of alcohol solvents on chelate formation,[3] we decided to study the effect of chain length and isomerism

in this type of solvent. For this purpose an indicator solution composed of 8.0 gm. of *n*-propyl gallate, 4.0 gm. of ferric chloride, 3.0 ml. of acetyl chloride, and 200 ml. of glacial acetic acid was prepared. This indicator solution, 0.1 ml., and 0.1 ml. of *o*-chloroaniline were added to 10.0 ml. of an alcohol sample previously fractionated over, or pretreated with, alkali. The CTT values were measured in the usual manner with a precision of better than 1° C. for primary

TABLE 4
CRITICAL THERMOCHROMIC TEMPERATURES OF ALCOHOLS

Alcohol*	CTT†	T_{ca}-T_{cp}‡	CTT (calc.)
Methanol	99.0	322.5	98.5
Ethanol	82.5	211.0	78.9
1-Propanol	71.4 ± 0.5	168.1	71.4†
2-Propanol	51.8 ± 1.2		
2-Methyl-1-propanol	72.8 ± 0.6	143.0	67.5
1-Butanol	65.5 ± 0.8	136.4	65.8
2-Methyl-2-propanol	50.4 ± 0.5		
2-Butanol	45.9 ± 1.2		
2,2-Dimethyl-1-propanol	72.5 ± 0.4		
3-Methyl-1-butanol	71.1 ± 0.5	119.2	62.8
1-Pentanol	60.5 ± 0.4	115.8	62.2
2-Methyl-2-butanol	56.4 ± 1.5		
1-Hexanol	59.3 ± 1.0	103.2	60.0
1-Heptanol	58.6 ± 0.6	98.1	59.1
2-Ethyl-1-hexanol	63.0 ± 0.6		
1-Octanol	57.5 ± 0.4	88.8	57.5§
1-Nonanol	58.1 ± 0.8	86.0	57.0
1-Decanol	56.2 ± 0.8	84.0	56.6
1-Hendecanol¶	53.8 ± 0.6		
1-Dodecanol¶	51.3 ± 1.1		

* The alcohols are arranged in order of increasing number of carbon atoms. In an isomeric group the order is that of decreasing CTT.

† In cases of the secondary and tertiary alcohols the unequivocal blue end point is preceded by a grayish-blue shade over a range of 5° C. or less. Due to this anomaly 5° C. might be a more realistic value of precision in comparing the CTT values of these isomers with those of primary alcohols.

‡ This column indicates the difference in critical temperatures between an alcohol and its parent paraffin. A few of the values were obtained by extrapolation and interpolation.

§ These two values were used to calculate the constants in the given empirical equation relating CTT and critical temperatures.

¶ The CTT values of these alcohols are not as reliable as the others because the liquids used boiled over a range of 4° C. In most of the other cases the liquids boiled over 0.5° C. range with a maximum of 1° C.

alcohols. It was noted that repetition of this measurement 10 to 15 times on a given solution produced a downward trend, lowering the average deviation to about 2° C. for primary alcohols. If the CTT is remeasured after a solution has stood several days, the difference in the measured CTT values may be as much as 10° C.

TABLE 4 lists the CTT values in a variety of alcohols together with some critical temperature data for the purpose of correlation. For the normal alcohols it was found that the CTT value was proportional to the difference between the critical temperatures of the alcohol and its parent paraffin. The

empirical equation relating these quantities is the following:

$$\begin{aligned} CTT &= 1.75\,(T_{ca} - T_{cp}) + 41.9 \\ T_{ca} &= \text{critical temperature of a normal alcohol} \\ T_{cp} &= \text{critical temperature of the parent paraffin} \end{aligned} \qquad (8)$$

Such a relationship seemed reasonable because both the CTT values and the critical temperature differences decreased with chain length. This observation

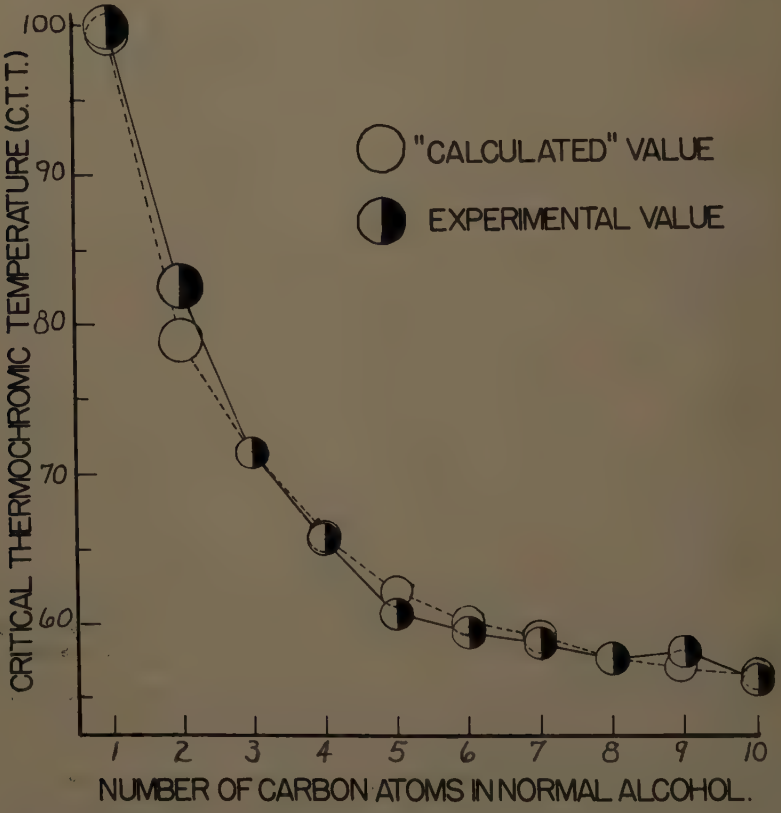


FIGURE 1. Variation of CTT as a function of chain length in the normal alcohols.

implied a decreasing importance of hydrogen bonding as an intermolecular force with chain lengthening. FIGURE 1 is a plot of the experimental CTT values of the normal alcohols and the values calculated from the above equation. Although the "experimental" values of methanol and ethanol were obtained by extrapolation of the dilution curves shown in FIGURE 2, it was apparent from the pale blue-green appearance of the ethanol solution at its boiling point that the chromic transition was only a few degrees higher. The methanol solution, on the other hand, showed no change in color intensity up to its boiling point.

At a given temperature the equilibrium concentration of the chelate is determined by the basicity of the medium. Since the concentration of *o*-chloroaniline is fixed and the chelate concentrations are the same at the CTT values, one may equate the basicities of the alcohols at those temperatures. This implies that the basicity order is the same as that of the CTT values. A comparison of these values for some isomeric alcohols (TABLE 4) shows the order of basicity to be: branched chain primary alcohols > normal alcohols > secondary and tertiary alcohols. This order is understandable in terms of a bulk alcohol

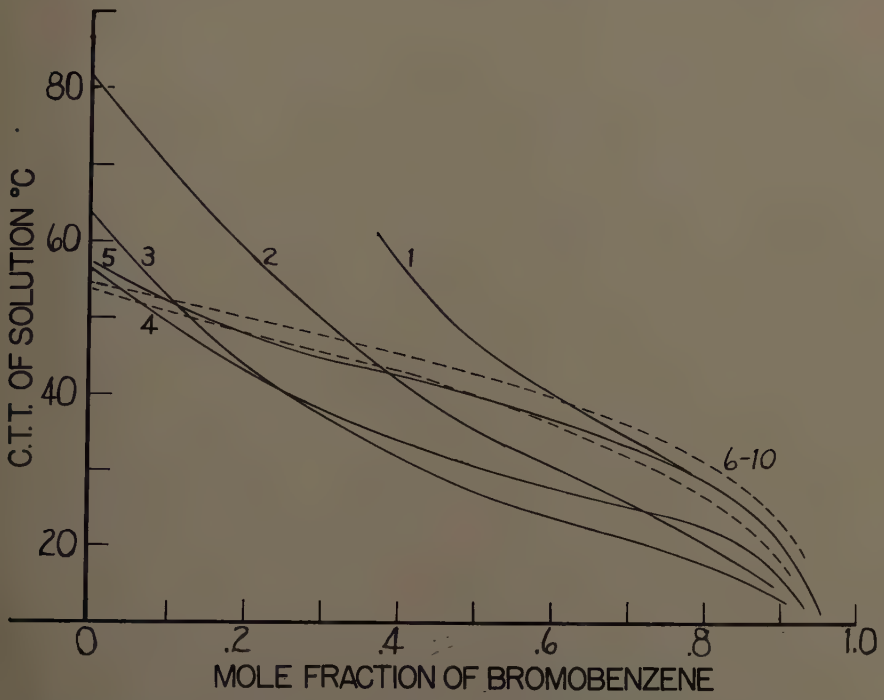


FIGURE 2. Depression of the CTT of the normal alcohols on dilution with bromobenzene The numbers refer to the number of carbon atoms in the alcohol. The dashed area is the enclosure of the curves for *n*-hexanol through *n*-decanol.

solvent containing molecules "polymerized" by hydrogen bonding in equilibrium with those "attached" by chain meshing. Since the formation of hydrogen bonds is the more exothermic process, the lower the temperature, the greater the concentration of hydrogen bonded polymers. In secondary and tertiary alcohols the steric effect of the alkyl groups surrounding the hydroxyl group inhibits hydrogen bonding in comparison with the normal isomer, thereby lowering their CTT values. Chain branching in a primary alcohol, on the other hand, reduces the efficiency of meshing without shielding the hydroxyl group, thus favoring a higher degree of hydrogen bonding. The decrease of the CTT with increasing chain length in the normal alcohols is a consequence of correspondingly increasing chain meshing (van der Waals) forces.

Since both the *o*-chloroaniline and the solvent contribute to the over-all basicity, the fixation of the former component leaves only the latter for consideration. The assumptions are made that the basicity of any bulk alcohol is dependent on the concentration of hydrogen bonded polymer P*, and that the latter is large with respect to the concentration of indicator.

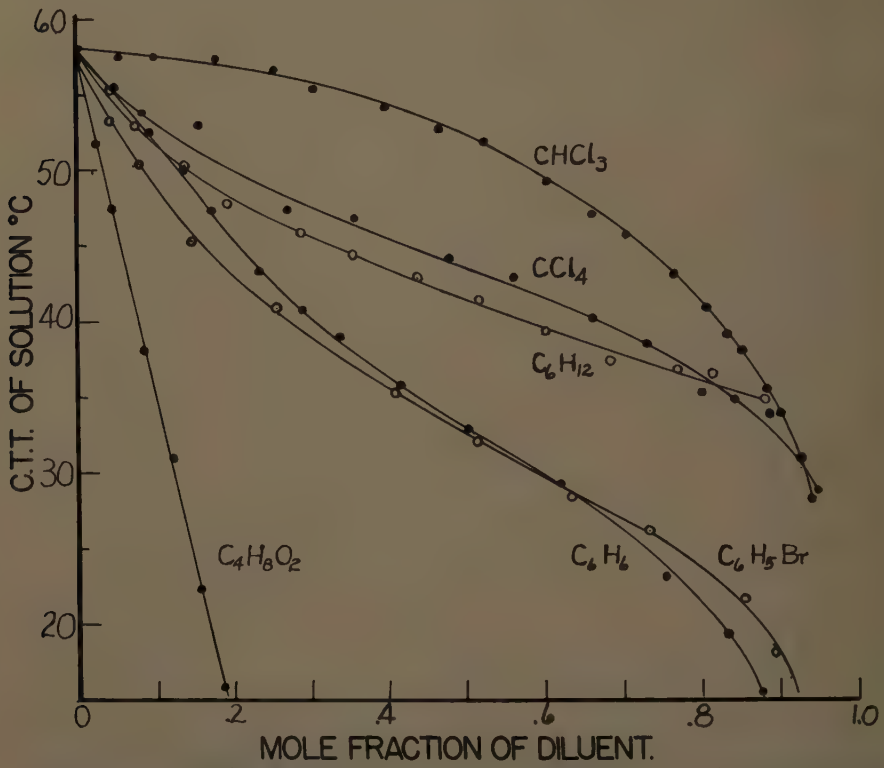


FIGURE 3. Depression of the CTT of *n*-butanol on dilution with the indicated liquids. $C_4H_8O_2$ is ethyl acetate and C_6H_{12} is cyclohexane.

$$P = \left[\begin{array}{l}\text{fractional number of hydrogen-}\\ \text{bonded oxygen atoms}\end{array}\right] \cdot \left[\frac{1600}{\text{molecular weight of an alcohol}}\right] \tag{9}$$

The equilibrium constant which measures the basicity of an alcoholic solvent is then given by **10**.

$$K_p = \frac{(A\text{-H-}P)}{(A\text{-H})(P)} \tag{10}$$

It follows that the observed constancy of the concentrations of conjugate in-

* Because of the probable presence of a distribution of different types of polymers, the concrete meaning of P is taken as the weight per cent of oxygen which is hydrogen bonded.

dicator base to acid at the CTT values in alcohols is due to equal P values. In turn, therefore, the K_p values are equated at those temperatures.*

In the following oversimplified treatment of the equilibrium **11** among the various species possibly present in alcohols only two types are considered: a hydrogen bonded dimer, P_h, and an "unassociated molecule", P_m.

$$2\,P_m \rightleftharpoons P_h \tag{11}$$

$$K = \frac{P_h}{P_m^2} = \frac{P_h}{(P_o - P_h)^2} \tag{12}$$

$$P_o = \text{maximum value of } P = \frac{1600}{\text{molecular weight of the alcohol}}$$

The application of the van't Hoff relationship to **12** for a given alcohol at the CTT and some other definite temperature yields **13**.

$$\ln\left[\frac{P_h}{(P_o - P_h)^2}\right] - \ln K_1 = \frac{\Delta H}{R}\left[\frac{1}{CTT} - \frac{1}{T_1}\right] \tag{13}$$

In order to test equation **13** for a homologous series the following assumptions are made:

(1) As stated previously, the P_h values for all solvents are identical at their CTT values.

(2) $P_0 \gg P_h$ at and above the CTT.

(3) The equilibrium constants, defined by equation **12**, are equal for all homologues at their atmospheric boiling points and critical temperatures.

(4) ΔH is a constant for all homologues over the range of temperatures considered.

For any alcohol, therefore, equation **13** reduces to **14**.

$$2 \ln P_o = \frac{\Delta H}{R}\left[\frac{1}{CTT} - \frac{1}{T_{bp}}\right] + \ln \frac{P_h}{K_{bp}} \tag{14}$$

T_{bp} = atmospheric boiling point

K_{bp} = equilibrium constant at T_{bp}

$\ln \frac{P_h}{K_{bp}}$ = constant (assumptions 1 and 3)

FIGURE 4 is a plot of $-\ln P_0$ against $[(1/CTT) - (1/T_{bp})]$. The slope of the line yields a value of 4.6 kcal. for the heat of hydrogen bonding, which compares favorably with the value of 6 kcal. obtained from infrared spectroscopy.[8] FIGURE 4 also contains a plot of $-\ln P_0$ against $[(1/CTT) - (1/T_{critical})]$. Another straight line is obtained from which the value of the heat of hydrogen bonding is 6.7 kcal. The difference in these values stems mainly from the assumption of the constancy of ΔH.

* In order to avoid any possible confusion or attempt at comparison of results, it must be emphasized that this work is unrelated to that of other investigators on the basicity of alcohols. Previous studies such as that of Pratt and Matsuda[7] have dealt with the relative basicity of dilute solutions of alcohols in other solvents and not with the bulk media themselves.

FIGURE 2 shows the depression of the CTT values of the normal alcohols on dilution with bromobenzene. FIGURE 3 is a plot of the depression of the CTT of *n*-butanol with the following diluents: chloroform, carbon tetrachloride, benzene, cyclohexane, and ethyl acetate. The desired diluent was delivered from two burettes in equal volumes, one containing the indicator solution and the other containing *o*-chloroaniline. The concentration of the indicator sys-

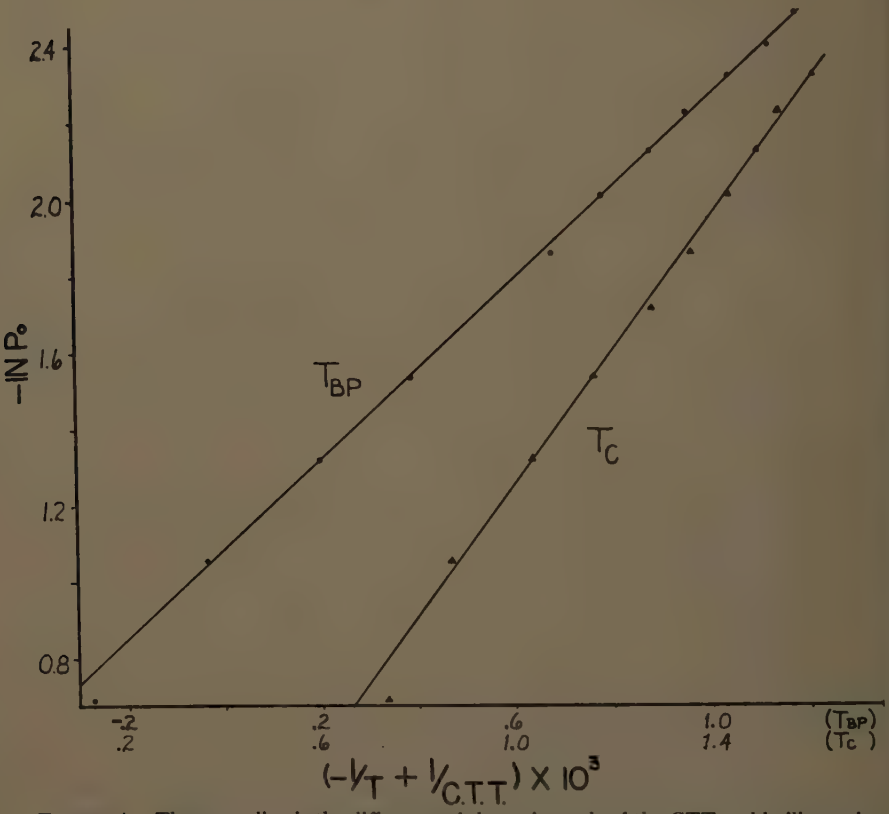


FIGURE 4. The upper line is the difference of the reciprocals of the CTT and boiling point at atmospheric pressure as a function of the negative logarithm of the atomic weight fraction of oxygen in the normal series of alcohols. The lower line shows the same relationship in which the critical temperature is substituted for the boiling point.

tem was constant throughout. The experiments were designed this way because precipitation of the chelate occurred in some of the diluents either immediately or on standing for less than the full time required for completion of the observations. In the case of the hydrocarbon diluents it was even necessary to add some of the alcohol under test to the diluent in order to prevent precipitation of some ferric complex.

These dilution curves are interpreted as the result of a competition between hydrogen bonded networks for protons liberated in chelate formation and "isolated" alcohol molecules for Fe(III). The addition of a diluent such as bromo-

benzene, benzene, hexane, or decane reduces the CTT by breaking up the network, which presumably promotes chelation by accepting protons from the indicator reactants. On the other hand the "isolated" molecules preferentially bind ferric chloride, thus inhibiting the reaction among the required components. The longer the chain of a given alcohol, the smaller the effect of a hydrocarbon or similar diluent. This observation may be ascribed to the van der Waals binding of the diluent by the chain, which is a function of length. The dilution curves of FIGURE 2 decrease in steepness with increasing chain

TABLE 5
CRITICAL THERMOCHROMIC TEMPERATURES OF MISCELLANEOUS COMPOUNDS

Compound	CTT*	Remarks
Aldehydes		
2-Ethyl hexaldehyde	Below† −63° C.	
o-Tolualdehyde	Below −36° C.	
Ketones		
Acetone	−87° to −92° C.	Yellow throughout liquid state Blue in solid state only
Acetophenone	15° C.	Same as acetone
Cyclopentanone	Below −40° C.	Green in solid state
3-Heptanone	Below −39° C.	
Esters of carboxylic acids		
Ethyl chloroacetate	Below −15° C.	Purple in the solid state
Methyl acetate	−88° to −92° C.	Same as acetone
Methyl caproate	Below −65° C.	
Methyl dichloroacetate	−13° C.	
Methyl myristate	Below 15° C.	Same as acetone
n-Propyl carbonate	−35° C.	Same as acetone
Ethers		
Diamyl ether	Below −62° C.	
Diethyl ether	Below −90° C.	
Diphenyl ether	Green at room temperature	Turned blue after 15 min.
Nitriles		
Caprylonitrile	Below −45° C.	
Heptanonitrile	Below −59° C.	
m-Tolunitrile	−15° C.	Color change to a gray-purple

* No attempt was made to investigate the entire liquid range in all cases.
† The observation that the CTT may be below a certain temperature should not imply its existence either in the liquid or solid states.

length so that there is very little change after *n*-pentanol. It should also be observed that all the curves tend to converge. This lends credence to the hypothesis that the "isolated" molecules, regardless of chain length, are equally effective in reducing the CTT.

Of some interest is the comparison of chloroform and carbon tetrachloride. Up to a mole fraction of 0.1 the former causes no change in CTT whereas the latter produces a drop of 5° C. This difference is probably due to the fact that chloroform can enter the alcohol polymeric network, possibly as an end group at low concentrations. The relative effectiveness of benzene and bromobenzene over cyclohexane may be due to their ability to pi-bond ferric chloride. On the assumption that esters function as strong Lewis bases toward ferric chloride,

the linear drop in CTT with increasing concentration of ethyl acetate is understandable.

It should be noted from TABLE 5 that the blue color of the chelate appears in some nonhydrogen bonded liquids only after solidification. This observation suggests that in the change of state there is a great increase in some polymeric structure which favors proton solvation. Since a decreasing temperature favors dipolar alignment, it may be that this orientation reaches a sudden maximum on solidification. Proton solvation may be one of charge resonance in a dipolar matrix. It is interesting that many aldehydes trimerize[9] at a greater rate in the solid than in the liquid state. This phenomenon must be due to the proper dipolar orientation of carbonyl groups within bonding distance in the solid state.

Acknowledgments

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INFRARED SPECTRA AND CORRELATIONS FOR THE ETHYLENEDIAMINETETRAACETIC ACID METAL CHELATES*

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The complexes formed between metal ions and ethylenediaminetetraacetic acid (hereafter referred to as EDTA) have been extensively studied in terms of their properties in solution.[1-6] However, until recently, little was known concerning the properties of these chelates in the solid phase. Also, the nature of the bonding, both in solution and in the solid phase, has been studied only to a limited extent during the last five years. An uncertainty has existed regarding the coordination number for many metal ions as well as for the EDTA ligand.[7]

The infrared absorption spectra for the metal-EDTA chelates have been found useful in establishing the character of the metal-ligand bonding. Busch and Bailar[8] first applied the infrared technique in the study of the Co(III)-EDTA complexes and demonstrated that the EDTA molecule can serve both as a sexidentate and a pentadentate ligand for Co(III). This work was continued more extensively by Morris and Busch[9] for the pentadentate EDTA chelates of Co(III); these workers also observed that the infrared data could be used to determine the covalent character of the metal-ligand bonding. Other infrared studies have been made of the EDTA chelates in which the ligand has been concluded to be sexidentate for the Cu(II) chelate[10] and both bidentate and quadridentate for Pd(II) and Pt(II) chelates.[11]

More recently a series of intensive studies has been made of the infrared spectra for the EDTA chelates of the alkaline earth ions,[12] of the divalent metal ions,[13] and of the higher valent metal ions.[14] Although specific conclusions were made in each of these studies, an over-all set of correlations between the properties of the metal-EDTA chelates and their infrared spectra was not possible until now. The present discussion is concerned with making such general correlations and with summarizing the infrared investigations of this group of metal-EDTA chelates.

Infrared spectra have been recorded for the EDTA chelates of Mg(II), Ca(II), Sr(II), Ba(II), Mn(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II), Hg(II), Pb(II), Al(III), Ce(III), Bi(III), V(III), V(IV), Cr(III), Fe(III), Co(III), Ti(IV), Th(IV), Mo(V), and Mo(VI), and are presented as a part of this discussion and summary. The interactions between these metal ions and the nitrogen and oxygen atoms of the EDTA anion are of interest and can be related to the infrared absorption spectra of the chelates. The use of the infrared technique to determine whether the bonding in EDTA chelates is primarily ionic or covalent was first suggested by Morris and Busch[9] and has been reviewed previously.[12-14] This approach has been applied to the above group of metal chelates as a means of determining the kind of bonding. Some limita-

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tion can also be given to the maximum number of EDTA-donor groups involved in chelation from the infrared data, but the actual number usually cannot be determined.

TABLE 1

MAJOR INFRARED ABSORPTION PEAKS FOR THE CH_2, COO^- AND CN GROUPS IN THE METAL-EDTA CHELATES*

Chelate	r, ionic radius	CH_2	COO^- antisymmetrical vibration	COO^- symmetrical vibration	Δ, frequency difference	CN	pK, stability constant
$Na_2MgY \cdot 4H_2O$	0.66	2920	1610	1425	185	1115	8.7
$Na_2CaY \cdot 3.5H_2O$	1.01	2840	1605	1420	185	1120	10.7
$Na_2SrY \cdot 5H_2O$	1.18	2840	1595	1415	180	1110	8.6
$Na_2BaY \cdot 1.5H_2O$	1.34	2830	1590	1410	180	1105	7.8
$Na_2MnY \cdot 2H_2O$	0.80	2920	1600	1420	180	1105	13.8
$Na_2CoY \cdot 1.5H_2O$	0.78	2950	1600	1395	205	1110	16.3
$Na_2NiY \cdot 4H_2O$	0.68	2950	1605	1400	205	1105	18.6
$Na_2CuY \cdot 1.5H_2O$	0.69	2930	1605	1390	215	1120	18.8
$Na_2ZnY \cdot 3.5H_2O$	0.70	2950	1600	1395	205	1110	16.5
$Na_2CdY \cdot 2H_2O$	0.92	2920	1595	1410	185	1105	16.5
$Na_2HgY \cdot 2.5H_2O$	1.05	2920	1595	1405	190	1105	21.8
$Na_2PbY \cdot 1H_2O$	1.17	2900	1600	1400	200	1100	18.0
		(2850)	(1645)	(1375)	(270)		
$NaAlY \cdot xH_2O$	0.45	2940	1570	1405	165	1095	16.1
$NaCeY \cdot 2H_2O$	1.02	2920	1600	1415	185	1100	16.0
$NaBiY \cdot 2H_2O$	1.16	2940	1610	1390	220	1095	—
			(1570)	(1440)	(130)		
$NaVY \cdot 5H_2O$	—	2960	1630	1405	225	1115	25.9
$Na_2VOY \cdot 5H_2O$	0.64	2960	1630	1390	240	1095	18.8
$NaCrY \cdot 2H_2O$	0.55	2980	1640	1360	280	1090	—
$NaFeY \cdot 1H_2O$	0.53	2980	1635	1385	250	1105	25.1
$NaCoY \cdot 2H_2O$	—	3000	1645	1370	275	1075	36
$TiOH_2Y \cdot H_2O$	0.64	2970	1620	1350	270	1085	—
$ThY \cdot 9H_2O$	—	2920	1630	1395	235	1095	23.2
			(1535)	(1410)	(125)		
$Na_2Mo_2O_4Y \cdot H_2O$	—	2970	1660	1385	275	1065	—
			(1630)	(1400)	(230)		
$Na_4Mo_2O_6Y \cdot 8H_2O$	—	2940	1630	1395	235	1070	—
			(1600)	(1420)	(180)		
$Na_4Y \cdot 0.5H_2O$	0.96	2800	1605	1410	195	1120	1.7
			(1575)	(1435)	(140)		
K_4Y	1.33	2800	1595	1405	180	1120	—
NaOAc	—	—	1580	1430	150	—	—

* A more extensive set of correlations and infrared data for this group of EDTA chelates can be found in references 12 to 14. Abbreviations used in the table: Y, ethylenediaminetetraacetate anion; Δ, frequency difference between the absorption peaks for the antisymmetrical and the symmetrical vibrations for the COO^- groups in EDTA; r, ionic radius, Ångström units, for the metal ions.[17,18] Frequencies for absorption peaks are given in wave numbers, $cm.^{-1}$. The pK for each chelate (if known)[1-6] is tabulated in the right column and represents the negative logarithm of the dissociation constant.

Experimental

The infrared spectra were recorded with a Perkin-Elmer model 21 recording spectrophotometer equipped with a sodium chloride prism. The solid chelates were pressed into disks using KBr as the diluent.[15] Approximately 1 to 3 mg. of the chelate was intimately mixed with 500 mg. of KBr for each disk.

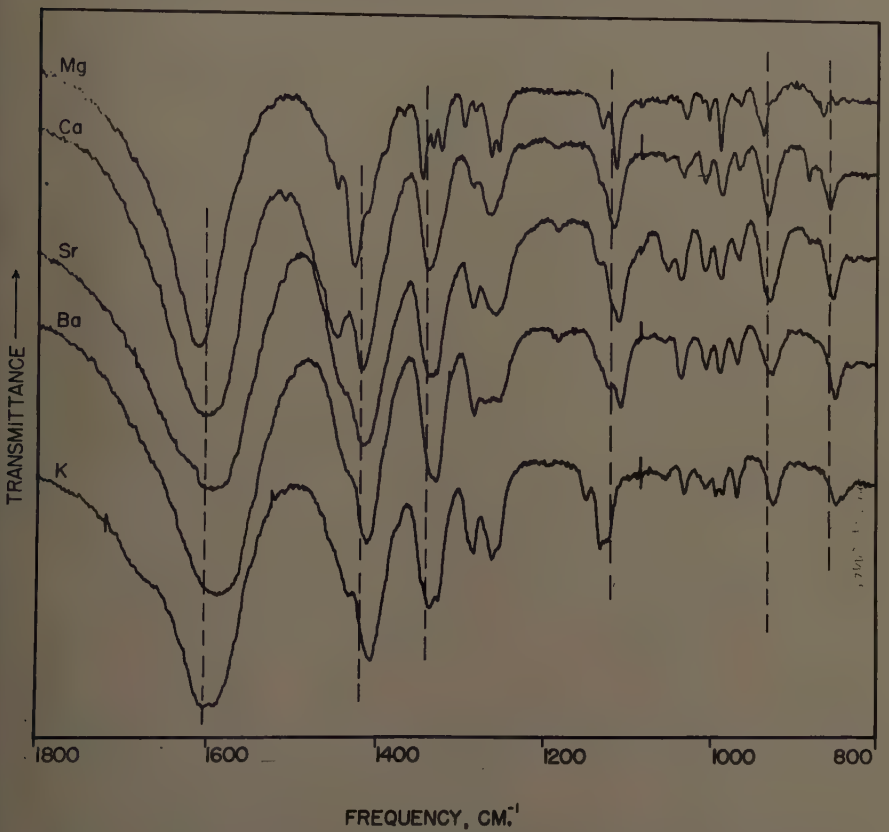

FIGURE 1. Infrared spectra of the EDTA chelates of the alkaline earth ions and of the tetrapotassium salt of EDTA: **Mg**, $Na_2[Mg(C_{10}H_{12}N_2O_8)]\cdot 4H_2O$; **Ca**, $Na_2[Ca(C_{10}H_{12}N_2O_8)]\cdot 3.5H_2O$; **Sr**, $Na_2[Sr(C_{10}H_{12}N_2O_8)]\cdot 5H_2O$; **Ba**, $Na_2[Ba(C_{10}H_{12}N_2O_8)]\cdot 1.5H_2O$; **K**, $K_4(C_{10}H_{12}N_2O_8)$.

The synthesis, isolation, and analysis of the metal-EDTA chelates have been discussed in detail previously[12-14] and will not be discussed here. The specific formulas for the various solid chelates are indicated in TABLE 1 and were determined by C-H microanalyses as well as by analysis for the metal ions. For comparison purposes the tetrasodium and tetrapotassium salts of EDTA have been prepared. Their infrared spectra have been recorded, as has the spectrum for sodium acetate; these data are useful in establishing the correlations between the properties of the metal-EDTA chelates and their infrared spectra.

Results and Discussion

The infrared spectra for the EDTA chelates of the four alkaline earth chelates are shown in FIGURE 1; the spectrum for the tetrapotassium salt of EDTA is shown at the top for reference. Guide lines have been placed on all of the figures to aid in comparing the spectra and the major absorption bands. In

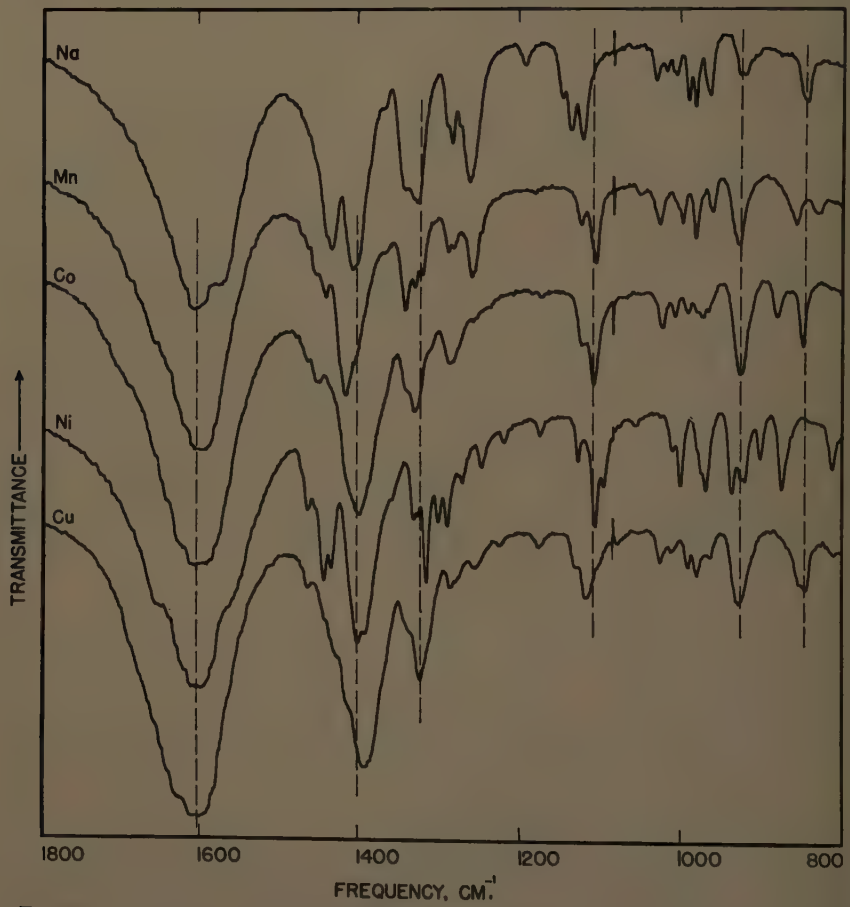

FIGURE 2. Infrared spectra of the EDTA chelates of Mn(II), Co(II), Ni(II), and Cu(II) and of the tetrasodium salt of EDTA: **Na**, $Na_4(C_{10}H_{12}N_2O_8) \cdot 0.5H_2O$; **Mn**, $Na_2[Mn(C_{10}H_{12}N_2O_8)] \cdot 2H_2O$; **Co**, $Na_2[Co(C_{10}H_{12}N_2O_8)] \cdot 1.5H_2O$; **Ni**, $Na_2[Ni(C_{10}H_{12}N_2O_8)] \cdot 4H_2O$; **Cu**, $Na_2[Cu(C_{10}H_{12}N_2O_8)] \cdot 1.5H_2O$.

FIGURE 2 are shown the infrared spectra for the EDTA chelates of Mn(II), Co(II), Ni(II), and Cu(II). Because these four ions all have unfilled *d*-subshells, comparison of their spectra as a group is of interest. For reference, the infrared spectrum of the tetrasodium salt of EDTA is shown at the top of FIGURE 2. The spectra for the EDTA chelates of Zn(II), Cd(II), Hg(II), and Pb(II) are shown in FIGURE 3 and illustrate the infrared characteristics for a group of divalent ions with filled *d*-subshells. In FIGURE 4 are shown the

spectra for the EDTA chelates of three trivalent ions that are not transition metals: Al(III), Ce(III), and Bi(III). The spectrum for the tetrasodium salt of EDTA is shown at the top of this figure for reference. The spectra for the EDTA chelates of V(III), V(IV), Cr(III), Fe(III), and Co(III) are shown in FIGURE 5 and illustrate the infrared characteristics of higher valent ions with unfilled *d*-subshells. In FIGURE 6 are shown the infrared spectra for the EDTA

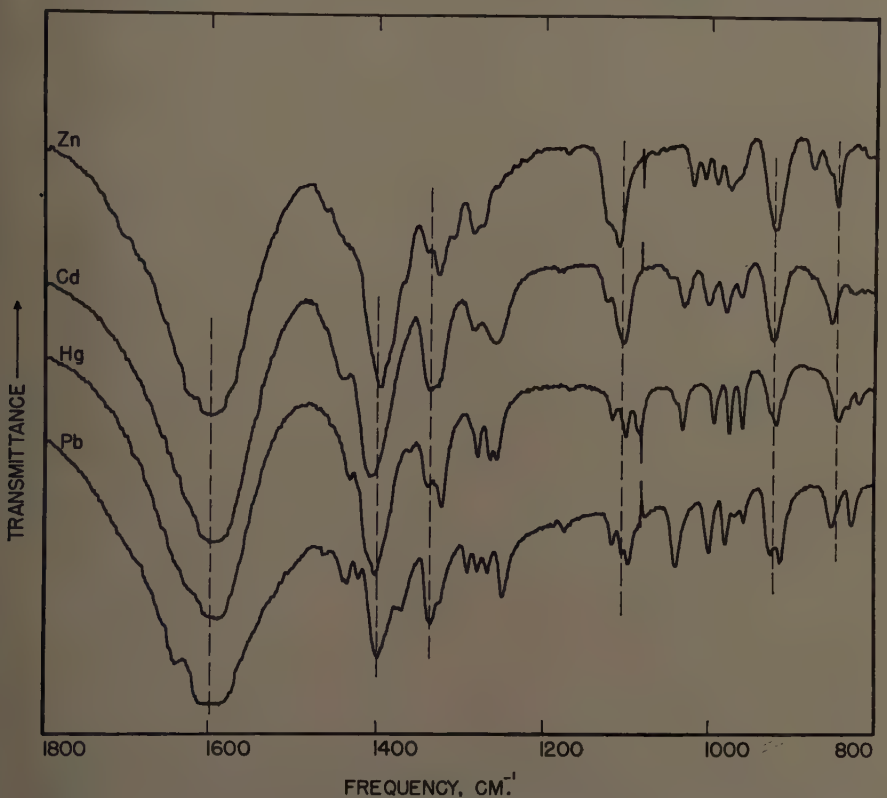

FIGURE 3. Infrared spectra of the EDTA chelates of Zn(II), Cd(II), Hg(II), and Pb(II): **Zn**, $Na_2[Zn(C_{10}H_{12}N_2O_8)]\cdot 3.5H_2O$; **Cd**, $Na_2[Cd(C_{10}H_{12}N_2O_8)]\cdot 2H_2O$; **Hg**, $Na_2[Hg(C_{10}H_{12}N_2O_8)]\cdot 2.5H_2O$; **Pb**, $Na_2[Pb(C_{10}H_{12}N_2O_8)]\cdot 1H_2O$.

chelates of Ti(IV), Th(IV), Mo(V), and Mo(VI). The molybdenum chelates are unique because there are two metal ions per ligand in the complex.

In TABLE 1 assignments and wave numbers are listed for the major absorption peaks of the CH_2, COO^-, and CN groups in the metal-EDTA chelates. For comparison, similar data are given for the tetrasodium and tetrapotassium salts of EDTA, and for sodium acetate. The ionic radii for the metal ions and the stability constants for the chelates are also listed, if known.

The C—H stretching absorption band. The infrared absorption band at 2800–3000 cm.$^{-1}$ has been assigned[16] to the C—H stretching vibration in the CH_2 groups of the EDTA molecule. For each of the chelates listed in TABLE 1 the frequency of the C—H peak is in the region of 2830–3000 cm.$^{-1}$ and is

taken as strong evidence for the formation of a chelate. The acid and the sodium-acid salts of EDTA[12] show this peak at 3020 to 3030 cm.$^{-1}$, while the tetrasodium and tetrapotassium salts absorb at 2800 cm.$^{-1}$. The characteristic frequency for the CH_2 groups in chelated EDTA molecules suggests that the COO^- groups are attached directly to the metal ion.

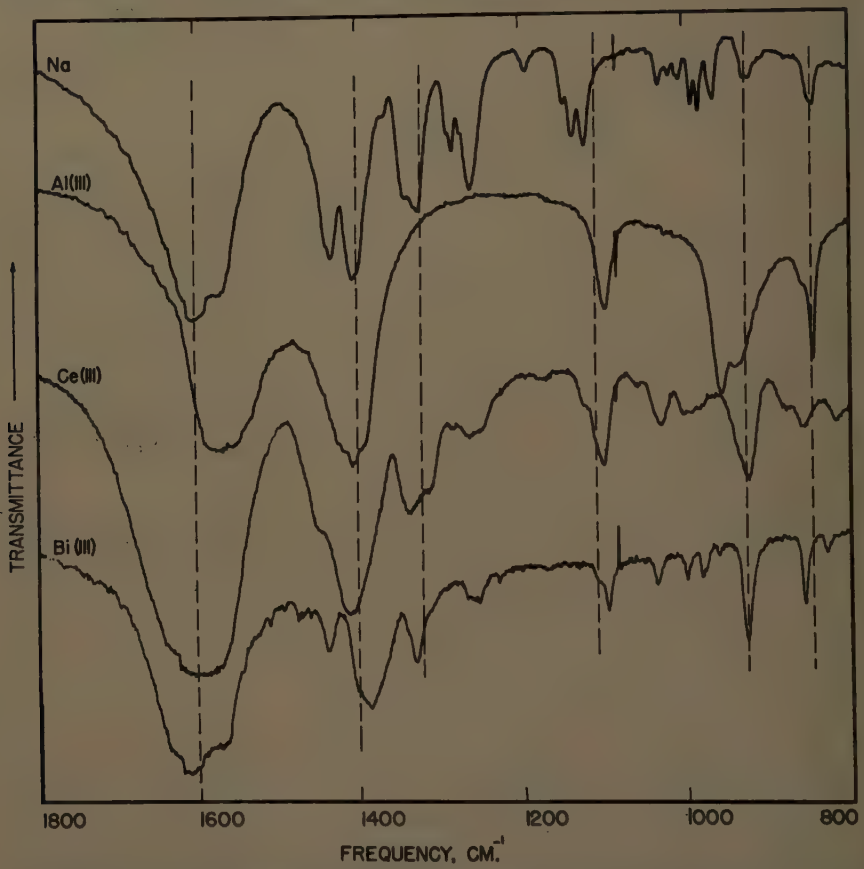

FIGURE 4. Infrared spectra of the EDTA chelates of Al(III), Ce(III), and Bi(III) and of the tetrasodium salt of EDTA: **Na**, $Na_4(C_{10}H_{12}N_2O_8)\cdot 0.5H_2O$; **Al(III)**, $Na[Al(C_{10}H_{12}N_2O_8)]\cdot xH_2O$; **Ce(III)**, $Na[Ce(C_{10}H_{12}N_2O_8)]\cdot 2H_2O$; **Bi(III)**, $Na[Bi(C_{10}H_{12}N_2O_8)]\cdot 2H_2O$.

As seen in TABLE 1, the frequency for the CH_2 group decreases generally as the ionic radius of the metal ion increases; this trend is particularly true for closely similar groups of ions, for example, the alkaline earth ions or the divalent ions. The shift of frequency with changes in ionic radius is also evidence that the metal ion has an effect on the vibration of the C—H bond and must be attached to the COO^- groups. This conclusion is further confirmed by the data for the tetrasodium and tetrapotassium salts of EDTA, which show the CH_2 absorption peak for both salts to be at 2800 cm.$^{-1}$. Thus, in the absence of chelate rings the frequency for the C—H absorption peak is 2800 cm.$^{-1}$;

as the chelate rings form and become increasingly tight, the frequency increases. For the weakest chelate in TABLE 1, Ba(II)-EDTA, the frequency is 2830 cm.$^{-1}$; for the strongest chelate, Co(III)-EDTA, the frequency is 3000 cm.$^{-1}$. Hence,

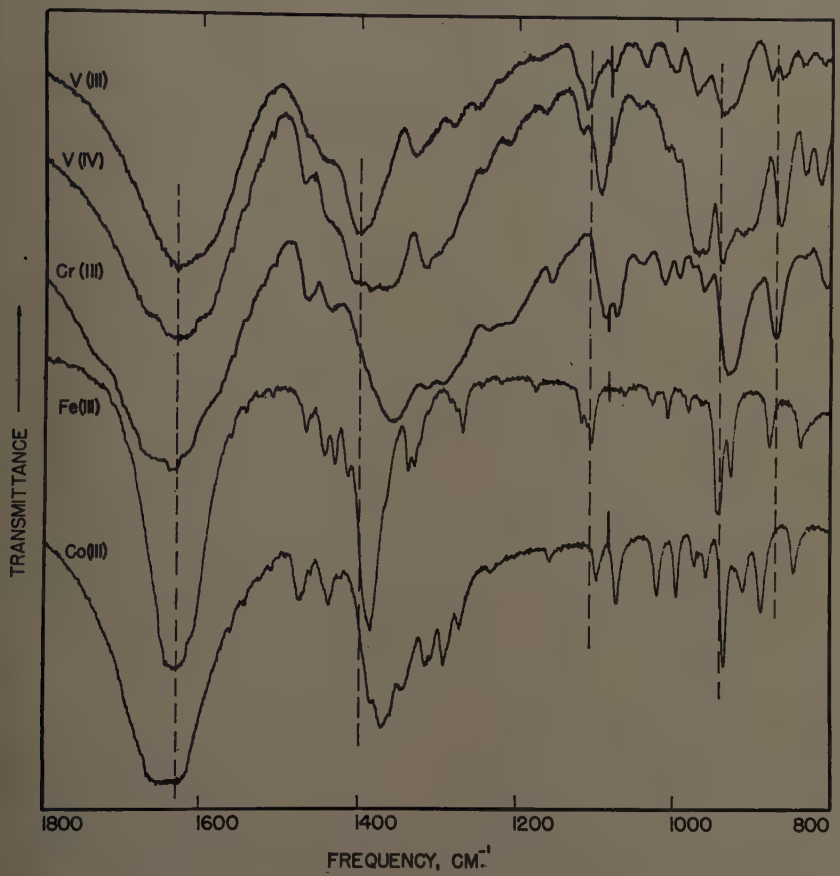

FIGURE 5. Infrared spectra of the EDTA chelates of V(III), V(IV), Cr(III), Fe(III), and Co(III): **V(III)**, $Na[V(C_{10}H_{12}N_2O_8)]\cdot 5H_2O$; **V(IV)**, $Na_2[VO(C_{10}H_{12}N_2O_8)]\cdot 5H_2O$; **Cr(III)**, $Na[Cr(C_{10}H_{12}N_2O_8)]\cdot 2H_2O$; **Fe(III)**, $Na[Fe(C_{10}H_{12}N_2O_8)]\cdot 1H_2O$; **Co(III)**, $Na[Co(C_{10}H_{12}N_2O_8)]\cdot 2H_2O$.

there is a rough correlation between the stability constant of the chelate and the frequency of the CH_2 peak.

The double peak for the Pb(II)-EDTA chelate may be evidence for some difference between the CH_2 groups. This could be accounted for by the presence of one or more carboxylate groups that are unbonded to the lead ion. An alternative conclusion would be that the bonding between the lead ion and the four COO^- groups of the EDTA ligand is different for one or more of the COO^- groups.

The antisymmetrical COO^- absorption band. The strongest and most char-

acteristic band for the carboxylate group (COO^-) is in the 1570 to 1610 $cm.^{-1}$ region, and is due to the antisymmetrical vibration of the COO^- group.[16] The COOH group gives a strong absorption band at 1675–1725 $cm.^{-1}$ due to C=O stretching; hydrogen bonding causes the frequency to decrease as double bond character is lost for the COOH group. Previous groups[8-11] have discussed the

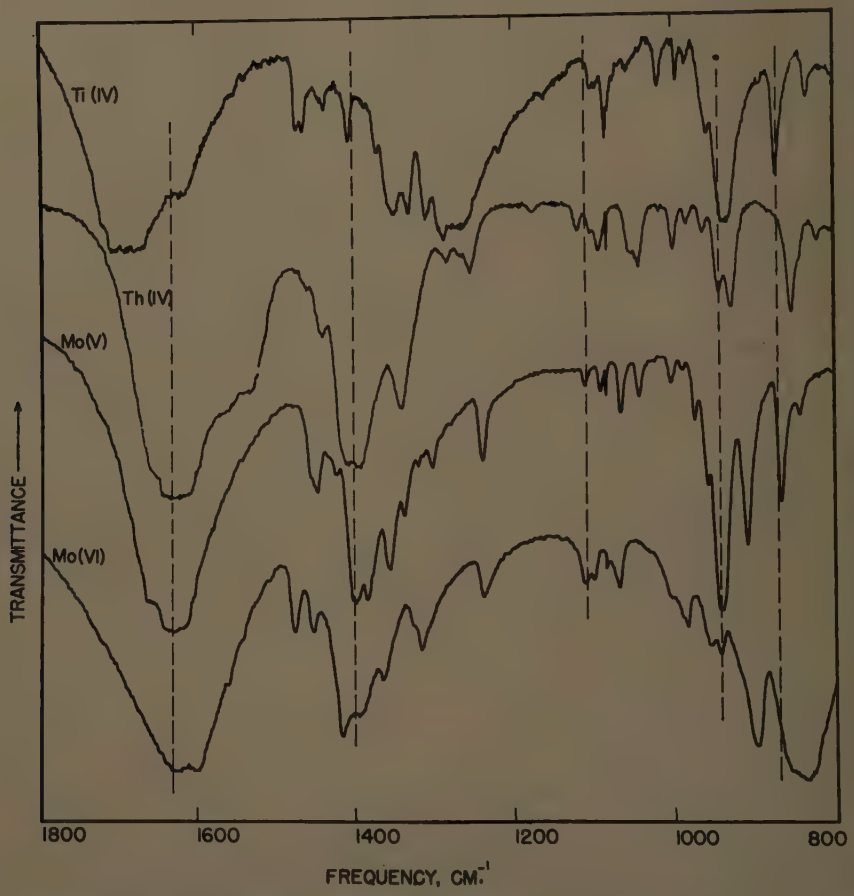

FIGURE 6. Infrared spectra of the EDTA chelates of Ti(IV), Th(IV), Mo(V), and Mo(VI): **Ti(IV)**, $TiO(C_{10}H_{14}N_2O_8)\cdot 1H_2O$; **Th(IV)**, $Th(C_{10}H_{12}N_2O_8)\cdot 9H_2O$; **Mo(V)**, $Na_2[Mo_2O_4(C_{10}H_{12}N_2O_8)]\cdot 1H_2O$; **Mo(VI)**, $Na_4[Mo_2O_6(C_{10}H_{12}N_2O_8)]\cdot 8H_2O$.

infrared spectra for the carboxylate groups of EDTA, particularly with reference to the EDTA chelates of Co(III), Cu(II), Pd(II), and Pt(II). Appearance of a separate peak in the 1675 to 1700 $cm.^{-1}$ region has been taken as evidence for the presence of carboxylic acid groups in the Co(III)-EDTA chelates,[8,9] in the Pd(II)-EDTA chelates, and in the Pt(II)-EDTA chelates.[11] Two groups[9,12] have used the frequency for the COO^- group in EDTA chelates as an indication of the covalent character for the metal-carboxylate bond.

The interpretation has been given that, as the bonding becomes more covalent, the frequency of the absorption peak increases; for Co(III)-EDTA,[9] which is considered to be covalently bonded, the frequency for the COO^- group is 1645 $cm.^{-1}$. When the frequency for this peak is 1610 $cm.^{-1}$ or less, the bonding is essentially ionic, as illustrated by the data for tetrasodium-EDTA and tetrapotassium-EDTA. Metal-EDTA chelates with frequencies for this peak of 1630 $cm.^{-1}$ or greater are concluded to be covalently bonded. As this peak increases in frequency, it approaches the frequency of the C=O stretching vibration (1700 $cm.^{-1}$), which would be expected for purely covalent bonding.

Reference to the data in TABLE 1 and to FIGURES 1, 2, and 3 indicates that the alkaline earth ions and all of the divalent metal ions are bonded ionically to the carboxylate groups of EDTA. Similarly, the data for the trivalent metal ions in FIGURE 4 indicate that they are bonded ionically to the COO^- groups. Reference to FIGURES 5 and 6 and to the corresponding data in TABLE 1 leads to the conclusion that the bonding is primarily covalent between the carboxylate groups of EDTA and higher valent ions of the transition metals.

The peak for the COO^- groups in the alkaline earth chelates (FIGURE 1) shows a decrease of frequency in the order: Mg > Ca > Sr > Ba. This corresponds to a decrease in frequency with increasing ionic radius and suggests that Mg has the most covalent character of the series and Ba the least, although all of these ions are ionically bonded primarily to the COO^- groups of EDTA. The trend in covalent character with a decrease in ionic radius is in agreement with the general rules favoring covalent bonds[19] and indicates that the order of increasing stability should be Ba < Sr < Ca < Mg.[20] This is in agreement with the stability constants determined in solution[1] except that Mg and Ca are reversed. The reversal for these ions can be attributed to the strong tendency for Mg to form hydrate bonds. When the complex is in the solid phase, water is not available, and Mg becomes the most strongly complexed by EDTA. Furthermore, the thermodynamic studies of the alkaline earth-EDTA complexes by Care and Staveley[21] show that the bonding between the carboxylate groups and the metal ion follow the order of strengths suggested by the infrared data: Mg > Ca > Sr > Ba.

Some complexing is indicated for the tetrasodium salt of EDTA; the frequency for the antisymmetrical COO^- vibration is greater than that observed for the Ba chelate or for the tetrapotassium salt. This conclusion is supported by the work of Schwarzenbach and Ackermann,[4] who have reported a stability constant for the EDTA complex of sodium ion ($\log K = 1.66$). Their work indicated that potassium ion is not complexed by EDTA; however, the relatively high frequency for the COO^- peak of K_4EDTA (greater than the Ba chelate and equal to the Sr chelate) would indicate that there is some interaction with the ligand.

The data for the chelates whose spectra are shown in FIGURES 2 and 3 indicate that the frequency for the COO^- peak generally decreases as the ionic radius increases. This is in agreement with the trends observed for the alkaline earth chelates. However, reference to the spectra for the chelates of the three ionically bonded trivalent metal ions (FIGURE 4) shows a reversal of this trend, especially in the case of the Al(III) chelate. The low frequency for this chelate might be accounted for if the ionic radius of Al(III) is so small (0.45Å)

that the metal-carboxylate bond is sterically hindered. The increase in frequency with increasing ionic radius in the case of the Ce(III) and Bi(III) chelates would suggest that the Bi(III) chelate has significant covalent character in spite of its large ionic radius.

The sharpness of the peak for the antisymmetrical vibration of the COO^- group has been used to support the assignment of six-coordinate bonding for Cu(II)-EDTA.[10] However, reference to FIGURES 1, 2, and 3 shows that all of these metal chelates except lead give a fairly sharp peak; in most cases it is as sharp as the peak for the Cu(II)-EDTA chelate. This suggests that all of these ions are six-coordinate. Such a conclusion is not warranted when it is noted that K_4EDTA also gives a sharp peak at 1595 $cm.^{-1}$. The only justifiable conclusion is that the appearance of a single sharp peak in the 1600-$cm.^{-1}$ region supports the possibility that the metal ion has a coordination number of six; it does not prove it. Thus, the alkaline earth ions may have a coordination number of six in their EDTA chelates that would be in agreement with the proposals of other investigators.[7] A similar suggestion of six-coordinate bonding is plausible, but not proved, from the infrared data for the EDTA chelates of Mn(II), Co(II), Cu(II), Zn(II), Cd(II), Hg(II), Al(III), and Ce(III).

The infrared method is particularly effective for indicating that one or more of the carboxylate groups of EDTA is not bonded to the metal; the presence of an extra peak in the 1600-$cm.^{-1}$ region is strong evidence for a coordination number less than six. This approach has been used effectively in the case of the Co(III)-EDTA chelates[8] and the Pd(II) and Pt(II) chelates.[11] Thus, the 1645 $cm.^{-1}$ shoulder for the Pb(II)-EDTA spectrum (FIGURE 3) could be attributed to the fact that one or more of the carboxylate groups is bonded to the lead ion. An alternative conclusion is that one or two of the metal-carboxylate bonds have some covalent character. The slight shoulder on the spectrum for the Ni(II) chelate (FIGURE 2) also suggests that one or more carboxylate groups of the EDTA molecule are different from the rest in this complex. However, in this case an X-ray study of the Ni(II)-H_2EDTA structure[22] has established that one of the COO^- groups is not bonded to Ni(II), but rather to a proton. Whether this is also the case for the more basic Ni(II)-EDTA chelate is not certain, but the tendency for having a free COO^- group is established. For the Bi(III)-EDTA spectrum (FIGURE 4), the shoulder at 1570 $cm.^{-1}$ indicates that one or more of the carboxylate groups probably is not bonded to the metal.

The broad bands or shoulders for the COO^- peak of the V(III), V(IV), Cr(III), Ti(IV), Th(IV), and Mo(VI) chelates (FIGURES 5 and 6) can be interpreted in two ways. Either the four carboxylates of the EDTA molecule are bonded to the metal ion in more than one way for these chelates, or one or more of the carboxylates of the EDTA molecule is not bonded to the metal. The latter alternative has been shown by Busch and Bailar[8,11] to be correct for the Pd(II), Pt(II), and Co(III) EDTA chelates, and seems reasonable for the Th(IV) chelate; from the spectrum it appears that one of the carboxylates is unbonded to the thorium ion. For the other five chelates either explanation is plausible. The spectrum for the Mo(VI) chelate shows a double peak in the 1600-$cm.^{-1}$ region; apparently, two of the COO^- groups are bonded to the

Mo(VI) ions one way (1630 cm.$^{-1}$ peak), and the other two (1600 cm.$^{-1}$ peak) are either bonded differently or are not bonded to the metal ions at all.

The Ti(IV) chelate has an infrared spectrum in the 1600- to 1700-cm.$^{-1}$ region (FIGURE 6) that shows three peaks (1705 cm.$^{-1}$, 1675 cm.$^{-1}$, and 1620 cm.$^{-1}$) that can be attributed to two COOH groups and to two COO^- groups. Both of the COO^{-1} groups may be bonded to the TiO^{++} ion or, possibly, only one of them. Thus, the infrared data support the conclusion that the correct formula for this complex is $TiOH_2EDTA \cdot H_2O$ rather than $TiEDTA \cdot 2H_2O$.

The sharp peak for the Fe(III), Co(III), and Mo(V) chelates in the 1600 cm.$^{-1}$ region suggests that all 4 carboxylate groups of the EDTA molecule are bonded to the metal ion in these complexes. The infrared study of Co(III)-EDTA chelates by Busch and Bailar[8] lends support to this conclusion. Furthermore, in the case of the Co(III) chelate this conclusion is verified by Weakliem and Hoard's X-ray study of the Co(III)-EDTA structure.[23] Their work establishes conclusively that all 4 carboxylates as well as the 2 nitrogen atoms of EDTA are bonded to the Co(III) ion.

The symmetrical COO^- absorption band. The symmetrical vibration for the COO^- group gives an absorption band in the 1450- to 1350-cm.$^{-1}$ region. Because of the multiplicity of peaks, this band is less useful for studying bond character than the antisymmetrical vibration band at 1600 cm.$^{-1}$. However, some rough correlations are possible by considering the major peak in this band, which is the frequency tabulated in TABLE 1. The trend of lower frequencies with increasing ionic radii is observed for the alkaline earth, Ce(III) and Bi(III) chelates, but this correlation is not observed for the remainder of the chelates. For the four chelates whose spectra are shown in FIGURE 2, the frequency of this peak decreases as the stability constant increases in magnitude. The decrease in frequency might also be attributed to the filling of the *d*-subshell. The Ni(II) and Pb(II) chelates exhibit considerable splitting for this infrared band, which is an aid in qualitative identification of the specific chelates. This characteristic also supports the conclusion that one or more of the COO^- groups is different in these two chelates. The general splitting of the symmetrical vibration band for all of the chelates makes it useful for identification purposes.

An interesting correlation is observed if the difference in frequency is taken between the major peak (or peaks) due to the antisymmetrical vibration and the symmetrical vibration for the COO^- groups. The major peaks and their differences are tabulated in TABLE 1; where two pairs of major peaks are present for a chelate, the second one is listed in parentheses. The peaks for sodium acetate are listed for comparison and illustrate a completely ionic COO^- group. The differences in frequencies range from 150 cm.$^{-1}$ for sodium acetate to 280 cm.$^{-1}$ for the Cr(III)-EDTA complex. Reference to TABLE 1 indicates that the frequency difference increases as the bonding between the metal ion and the carboxylate groups becomes more covalent. Thus, the Co(III) and Cr(III) chelates, which are considered to have the most covalent bonding, have the largest frequency differences. From this correlation the bonding for the Al(III) chelate appears to be almost as ionic as it is for sodium acetate. By using the frequency difference as the criteria for the degree of covalent character for the metal-carboxylate bond, an arbitrary designation can be made that the bonds

are primarily covalent when the difference is 225 cm.$^{-1}$ or more. When the difference is less than 225 cm.$^{-1}$, the bonding is primarily ionic. Referring to TABLE 1, the metal chelates down through Bi(III) appear essentially to be ionically bonded; the remainder of the chelates essentially are covalently bonded. The Bi(III) and V(III) chelates are borderline, but the data for the 1600-cm.$^{-1}$ peak suggest that the division should come between these 2 metal ions. The frequency difference for the Mo(V) chelate indicates that its bonding is considerably more covalent than it is for the Mo(VI) chelate. This conclusion suggests that the oxygen atoms of the Mo(VI)-chelate molecule satisfy most of the coordination sphere for the metal ions.

Other COO$^-$ absorption bands. Another band due to the COO$^-$ groups of the EDTA chelates appears at 1365–1285 cm.$^{-1}$ in the spectra of FIGURES 1 to 6. For the alkaline earth chelates (FIGURE 1) the trend of decreasing frequencies with increasing ionic radii is again confirmed for this peak. This also corresponds to a trend of decreasing frequency as the stability constant decreases in magnitude. The chelates in FIGURE 2 have an opposite trend of decreasing frequency for this peak with decreasing ionic radii and with increasing stability constants. For the chelates in FIGURE 3 there is a comparable correlation between frequency and stability constant; however, the correlation between ionic radii and frequency ceases. The Ce(III) and Bi(III) chelates (FIGURE 4) follow the correspondence between frequency and stability constant, but no apparent correlations exist between this absorption peak and the remainder of the complexes whose spectra are shown in FIGURES 4, 5, and 6. The absence of a peak in this band for the Al(III) chelate is interesting, and is not readily explained.

Four addition absorption bands that may be due to the COO$^-$ groups are observed in the spectra of FIGURES 1 to 6: at 1285 to 1160 cm.$^{-1}$, 1055–995 cm.$^{-1}$, 990–900 cm.$^{-1}$ and 890–810 cm.$^{-1}$. No significant correlations can be made for these peaks, but they are useful for identification purposes. The band at 1285–1160 cm.$^{-1}$ may be due to the C—N bond rather than to the COO$^-$ group; spectra for ethylenediamine chelates frequently have an absorption band in this region. Also, metal-acetate salts generally do not exhibit a peak in this region.

The CN absorption band. The remaining peak tabulated in TABLE 1 has been assigned to the C—N bond and is observed in the range of 1145 to 1065 cm.$^{-1}$. Although the CN group has not been studied extensively by infrared techniques, the available data indicate a peak in the 1100 cm.$^{-1}$ region.[16,24] The metal acetates do not have a peak in this region, but the ethylenediamine chelates all show such a peak. Kirschner[10] has noted this peak in his studies of the Cu(II)-EDTA chelate. This peak is completely absent for the disodium salt of EDTA, which may be due to the formation of "zwitter" ions in this salt;[7] the closest peak is at 1055 cm.$^{-1}$. The trisodium salt of EDTA also exhibits the 1055-cm.$^{-1}$ peak in addition to a peak at 1125 cm.$^{-1}$; with the tetrasodium salt there are peaks only in the 1100-cm.$^{-1}$ region.

All of the chelates listed in TABLE 1 (and FIGURES 1 to 6) exhibit this peak for the CN groups in the EDTA molecule. Because there is frequent splitting of the absorption band, several of the resulting peaks can be considered in the study of the bonding for the chelates. The major peak for this band is tabu-

lated in TABLE 1. By using the peak with the highest frequency in the 1100-cm.$^{-1}$ region, there is a general correlation between the frequency and the ionic radii for all of the chelates in FIGURES 1 to 4 except Al(III); as the ionic radius increases, the frequency decreases. Apparently, for this group of ionically bonded EDTA chelates the formation of the chelate ring and its resultant geometry have a direct effect on the C—N bond; the smaller ions cause the frequency to be higher. Because of the small radius of Al(III) (0.45Å), steric effects may prevent ring closure and adherence to this correlation for the Al(III) chelate. Reference to the data in TABLE 1 for the tetrasodium salt of EDTA indicates that the sodium ion does not follow the correlation observed for the ionically bonded chelates. Although the ionic radius for sodium is greater than that for Cd(II), the frequency is some 10 cm.$^{-1}$ higher for the CN peak of Na_4EDTA than for Cd(II)-EDTA. This inconsistent behavior probably can be attributed to the absence of chelate rings in the sodium salt. The frequencies for the 2 major peaks exhibited by the sodium salt should represent the C—N bond in the absence of chelate effects. The absence of significant differences in frequency between the tetrapotassium and tetrasodium salts confirms this supposition, especially in view of the marked difference in ionic radii for the 2 ions.

Only 1 significant peak is observed in the 1100-cm.$^{-1}$ region for those metal ions that have little or no tendency to form metal-nitrogen coordinate bonds, with the exception of Mg(II). Thus, the metal chelates in FIGURES 1 and 4 exhibit this single peak, while the chelates in FIGURES 2, 3, 5, and 6 show considerable splitting in this band, particularly the covalently bonded chelates. The frequency for the major peak of the covalently bonded chelates (FIGURES 5 and 6) in the 1100 cm.$^{-1}$ region appears to decrease as the bonding becomes more covalent. If this conclusion is correct, then the Mo(V) chelate must be highly bonded covalently.

For the alkaline earth EDTA-chelates, Care and Staveley[21] have suggested that the calcium ion has the ideal size for the formation of chelate rings with the nitrogen atoms of EDTA. Therefore, calcium has the largest stability constant for aqueous EDTA solutions of the alkaline earth ions. This increased stability is attributed to the metal-nitrogen bond, and the data in TABLE 1 for the major peak due to the CN group tend to support this proposal. The order of decreasing frequency is Ca > Mg > Sr > Ba, which is the order of decreasing stability constants for the EDTA complexes.

It has been established that the strong peak at 840 cm.$^{-1}$ for the Mo(VI) chelate is due to the Mo—O bond.

Conclusion

The infrared data for the COO^- absorption peaks support the conclusion that the bonding is primarily ionic for the EDTA chelates of Mg(II), Ca(II), Sr(II), Ba(II), Mn(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II), Hg(II), Pb(II), Al(III), Ce(III), and Bi(III). Similar data for the V(III), V(IV), Cr(III). Fe(III), Co(III), Ti(IV), Th(IV), Mo(V), and Mo(VI) EDTA chelates lead to the conclusion that for these complexes the bonding is primarily covalent. Thus, a peak for the antisymmetrical vibration of the COO^- group in the 1610-

to 1550-cm.$^{-1}$ region is evidence for ionic bonding; when this peak is in the 1660- to 1630-cm.$^{-1}$ region this is evidence for covalent bonding.

A single sharp peak in the 1600-cm.$^{-1}$ region supports the possibility of a coordination number of six for the metal ions and the EDTA ligand, but does not prove it. Thus it is possible, but not established, that all of the metal ions considered here, with the exception of Ni(II), Pb(II), Bi(III), Ti(IV), Th(IV), and Mo(VI), are six-coordinate. This conclusion is particularly warranted in the case of the Fe(III), Co(III), and Mo(V) chelates, which have a sharp peak in the 1660- to 1630-cm.$^{-1}$ region.

The infrared spectra are especially useful in demonstrating that one or more of the carboxylate groups of EDTA is not bonded to the metal ion, or is bonded differently to it. Thus, the shoulders in the 1600-cm.$^{-1}$ region for the Ni(II), Pb(II), Bi(III), Th(IV), and Mo(VI) chelates indicate that 1 or more of the COO^- groups probably is not bonded to the metal ion. For the Ti(IV) chelate, 2 of the COO^- groups have protons; thus the chelate has the formula $TiOH_2EDTA \cdot H_2O$.

The difference in frequency between the major peak for the symmetrical vibration (1450 to 1350 cm.$^{-1}$) and the peak for the antisymmetrical vibration (1660 to 1570 cm.$^{-1}$) of the COO^- group indicates the degree of covalent bonding for the EDTA chelates. The frequency difference increases as the bonding becomes more covalent; for chelates for which the difference is 225 cm.$^{-1}$ or greater, the bonding is concluded to be primarily covalent. If the difference is less than 225 cm.$^{-1}$, the bonding is primarily ionic.

The C—H stretching peak (2800–3000 cm.$^{-1}$) for the CH_2 groups in the EDTA molecule is useful in establishing the formation of chelates. Appearance of this peak in the 3000- to 2830-cm.$^{-1}$ region is strong evidence for the formation of a chelate.

Correlations have been made for other functional groups and their resulting absorption peaks. Because solvent effects are absent, the infrared technique has specific advantages over solution studies. The EDTA chelates for this group of metal ions have been synthesized and isolated.

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FORMATION OF POLYNUCLEAR COMPLEXES IN AQUEOUS SOLUTION*

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It is well known that highly charged metal ions undergo polymerization in aqueous solution by the formation of bridges through oxo or hydroxo groups. The tendency to form complex aggregates may be reduced considerably in many cases by chelating the metal ions with suitable polydentate ligands. The degree of formation of polynuclear species is thus reduced because (1) the positive charge of the metal is lowered, and (2) the number of coordination sites available for olation is reduced.

The initial steps in the hydrolysis and olation of a wide variety of chelated and unchelated metal ions may be represented by the following equations:

$$MA(H_2O)_x \rightleftharpoons M[OH]A(H_2O)_{x-1} + H^+ \qquad (1)$$

$$K_a = \frac{[M[OH]A][H^+]}{[MA]} \qquad (1a)$$

$$2MA(H_2O)_x \rightleftharpoons (M[OH]A(H_2O)_{x-2})_2 + 2H^+ + 2H_2O \qquad (2)$$

$$K_D = \frac{[(M[OH]A)_2][H^+]^2}{[MA]^2} \qquad (2a)$$

$$2M[OH]A(H_2O)_{x-1} \rightleftharpoons (M[OH]A(H_2O)_{x-2})_2 + 2H_2O \qquad (3)$$

$$K_d = \frac{[(M[OH]A)_2]}{[(M[OH]A)]^2} \qquad (3a)$$

Here A represents the dissociated form of the ligand and [OH] represents a coordinated hydroxo group. Studies on a series of related cupric diamine chelates[1] have shown that in general the tendency to hydrolyze and polymerize increases as the stability of the diaquo chelate decreases, provided no unfavorable steric effects are involved. Thermodynamic constants pertaining to reaction 3 are listed in TABLE 1. Since values of log K_d are determined from the differences of two large quantities according to the equation

$$\log K_d = 2pK_a - pK_D ,$$

the values of the thermodynamic constants calculated from K_d values are only approximate. However, they show small, sometimes positive values of $\Delta H°$ and relatively large positive values of $\Delta S°$, indicating that the driving force in the formation of the dimer from the monohydroxo chelate compound is primarily an entropy effect. The increase in entropy with dimerization is due for the most part to the negative entropy contribution of the coordinated water molecules in the hydroxo chelate, and to the entropy increase that results when the restraint on these water molecules is removed as they are released to the solvent when dimerization occurs.

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Of considerable interest are chelates of ferric ion. Schwarzenbach and Heller[2] have shown that the first and second hydrolysis constants of Fe(III)-ethylenediaminetetraacetic acid (EDTA) at 20° C. in 0.1 *M* KCl have values of $10^{-7.49}$ and $10^{-9.41}$, respectively, but have made no mention of the possibility of dimerization of the monohydroxo chelate. A priori one might assume that dimerization is unlikely, since the metal ion is completely coordinated by six donor groups. However, displacement of one of the weakly basic acetate groups from the hydroxo chelate would permit the formation of polynuclear chelates to occur. Titration curves of Fe(III)-EDTA (FIGURE 1) exhibit *p*H depressions with increasing concentration in the buffer region corresponding to the first hydrolysis step, suggesting that some type of polymerization reaction is occurring.

TABLE 1

VALUES OF $\Delta H°$ AND $\Delta S°$ FOR DIMERIZATION REACTIONS OF Cu(II)-DIAMINE HYDROXO CHELATES

Ligand	$\Delta H°$ (kcal./mole)	$\Delta S°$ (cal./mole deg.)
N,*N*′-dimethylethylenediamine	+1.0	+21
N,*N*,*N*′,*N*′-tetramethylethylenediamine	+3	+28
N-hydroxyethylethylenediamine	+4	+23
N,*N*′-dihydroxyethylethylenediamine	−1	+3
α,α′-dipyridyl	−<0.6	+>21
o-phenanthroline	−<0.4	+>9

Combination of equations **1a**, **2a** and the material balance equations,

$$T_M = [MA] + [M(\mathrm{OH})A] + 2[(M(\mathrm{OH})A)_2] \qquad (4)$$

and

$$T_{\mathrm{OH}} + [\mathrm{H^+}] - [\mathrm{OH^-}] = [M(\mathrm{OH})A] + 2[(M(\mathrm{OH})A)_2], \qquad (5)$$

leads to the equation

$$[\mathrm{H^+}](T_{\mathrm{OH}} + [\mathrm{H^+}] - [\mathrm{OH^-}])/[MA] = K_a + 2K_D[MA]/[\mathrm{H^+}]. \qquad (6)$$

Here T_M is the molar concentration of metal ion and T_{OH} is the concentration of added hydroxide ion. If a dimer is formed in the region $m = 0 - 1$, a straight line should be obtained when $[\mathrm{H^+}](T_{\mathrm{OH}} + [\mathrm{H^+}] - [\mathrm{OH^-}])/[MA]$ is plotted as ordinate versus $2[MA]/[\mathrm{H^+}]$ as abscissa. The slope of this line is equal to K_D, and the intercept at $2[MA]/[\mathrm{H^+}] = 0$ is equal to K_a. As may be seen in FIGURE 2, a straight line was obtained at $t = 25°$ in 1 *M* KCl corresponding to the following values of the equilibrium constants: $pK_a = 7.58$; $pK_D = 12.21$; $\log K_d = 2.95$. Similar results obtained at t = 0.6° give: $pK_a = 7.97$; $pK_D = 12.71$; $\log K_d = 3.22$. Calculation of $\Delta H°$ and $\Delta S°$ for the dimerization reaction **3** gives values of −4.1 kcal./mole and −0.2 cal./mole deg., respectively. In contrast to the cases of dimerization of Cu(II)-diamine chelates, the dimerization of Fe(III)-EDTA proceeds only because of a favorable enthalpy change. The considerably lower value of $\Delta S°$ in the latter case is to be expected since no water molecules are released upon reaction of 2 moles of

monohydroxo chelate to form 1 mole of dimer. Since the values of the thermodynamic constants are based on data obtained at only 2 temperatures, the results must be regarded as tentative, and further experiments will be performed at various temperatures.

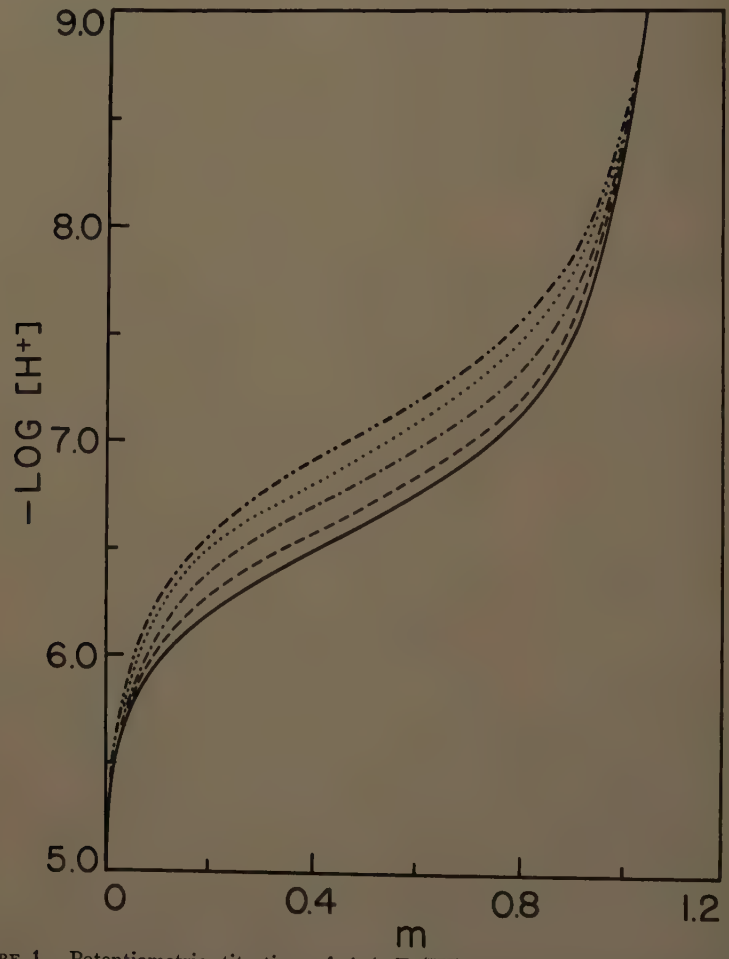


FIGURE 1. Potentiometric titration of 1:1 Fe(III)-EDTA chelate. Concentrations: ——, 8.5×10^{-2} M; – – – –, 5.6×10^{-2} M; –·–·–, 3.0×10^{-2} M; ·····, 1.6×10^{-2} M; – – ·– –, 8.2×10^{-3} M. Key: m = moles of base added per gram ion of Fe(III); $m = 0$ corresponds to complete formation of normal Fe(III)-EDTA chelate; $t = 25.0°$ C.; $\mu = 1.00$ (KCl).

It is of interest to compare the relative hydrolysis and olation tendencies of EDTA chelates of Zr(IV) (B. J. Intorre and A. E. Martell, unpublished results), Th(IV),[3] and Fe(III) shown in TABLE 2. Although the degree of basicity of these chelates increases in the order Zr < Th < Fe, the dimerization constants at 25° increase in the order Fe < Zr < Th. The low value

of K_d for Fe(III)-EDTA is probably due to the fact that the acetate groups must be displaced by the bridging hydroxo groups and to the fact that the charge on the central atom is less than in the cases of the Th(IV) and Zr(IV) chelates. The lower tendency toward dimer formation of the Zr(IV) chelate relative to that of Th(IV) may be due to steric hindrance caused by the small size of the central zirconium atom.

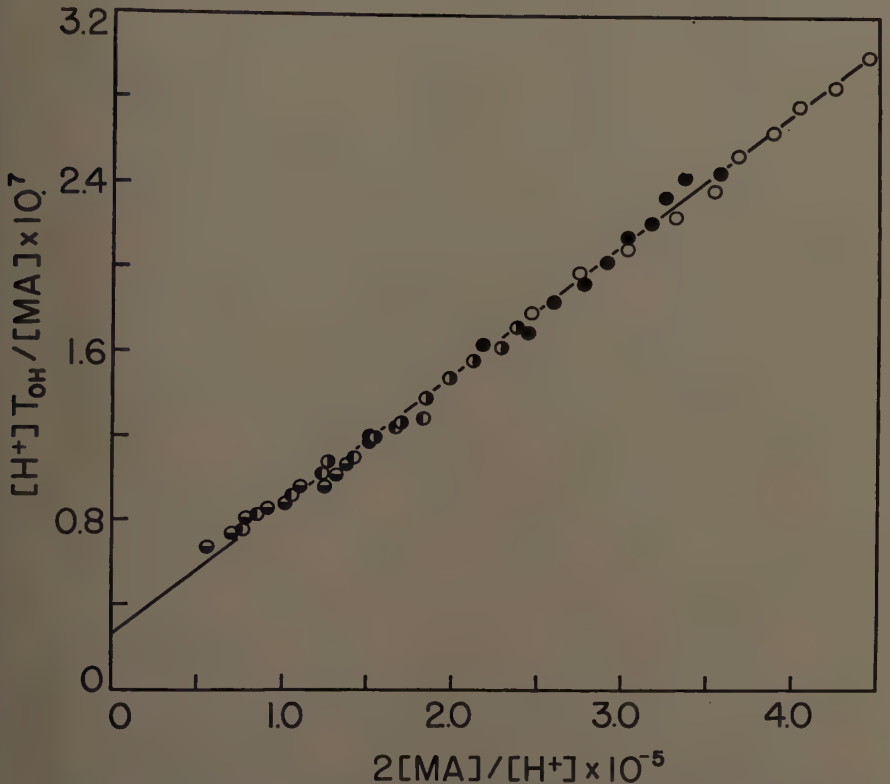


FIGURE 2. Plot of data of FIGURE 1 illustrating presence of the binuclear olated species Fe EDTA[OH]$_2$ Fe EDTA. Points calculated from data obtained at the following concentrations: ○, 8.5×10^{-2} M; ●, 5.6×10^{-2} M; ◑, 3.0×10^{-2} M; ◐, 1.6×10^{-2} M; ◒, 8.2×10^{-3} M.

TABLE 2
HYDROLYSIS AND OLATION OF EDTA CHELATES AT 25° C.

Metal	pK_a	pK_D	log K_d
Zr(IV)*	6.2	8.9	3.5
Th(IV)†	7.04	9.82	4.3
Fe(III)‡	7.58	12.21	2.95

* In 0.1 M KCl.
† In 0.1 M KNO_3.
‡ In 1.0 M KCl.

Titration of equimolar amounts of Fe(III) and *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA) results in a titration curve having a steep inflection after the addition of four moles of base per mole of metal chelate. This corresponds to the formation of a monohydroxo chelate of the type $FeAO^{1-}$ or $Fe[OH]AOH^{1-}$ (and corresponding polymerization products) where AOH^{3-}

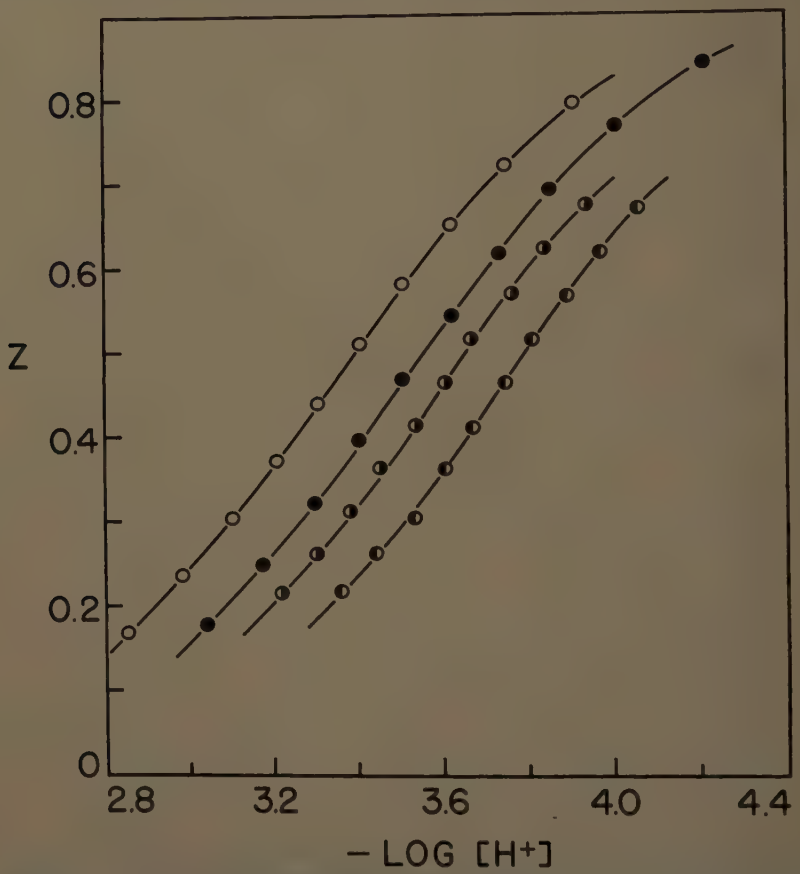


FIGURE 3. Plot of average number of hydroxo groups bound per mole of metal ion versus $-\log[H^+]$ for 1:1 Fe(III)-HEDTA chelates at various concentrations: ○, 7.3×10^{-2} *M*; ●, 4.0×10^{-2} *M*; ◑, 2.5×10^{-2} *M*; ◐, 1.3×10^{-2} *M*. t = 25.0° C. μ = 1.00 (KCl).

represents the triacetate anion of HEDTA. Sillen[4,5] and Hietanen and Sillen[6] in their treatment of polynuclear complexes have shown that in many cases a plot of $Z = T_{OH} + [H^+] - [OH^-]$ as ordinate versus $-\log [H^+]$ as abscissa produces a family of parallel curves for potentiometric titration data obtained at various metal ion concentrations. A plot of $-\log T_M$ versus $-\log [H^+]$ for data obtained at constant Z values then yields a straight line plot, the slope of which is equal to "l" in the general "core plus links" type complex, $M(M[OH]_t)_n$. As may be seen in FIGURE 3 a plot of Z versus $-\log [H^+]$ for Fe(III)-HEDTA

in the region $m = 3$ to 4 produces a series of curves that are essentially parallel. The plots of FIGURE 4 show that an average value of $(\partial \log T_M/\partial \log [H^+])_Z = 2.04$ is obtained, suggesting that a polymer of the general type $ThAOH \cdot (Th[OH]_2AOH)_n$ is obtained. The only polymer that is consistent with the

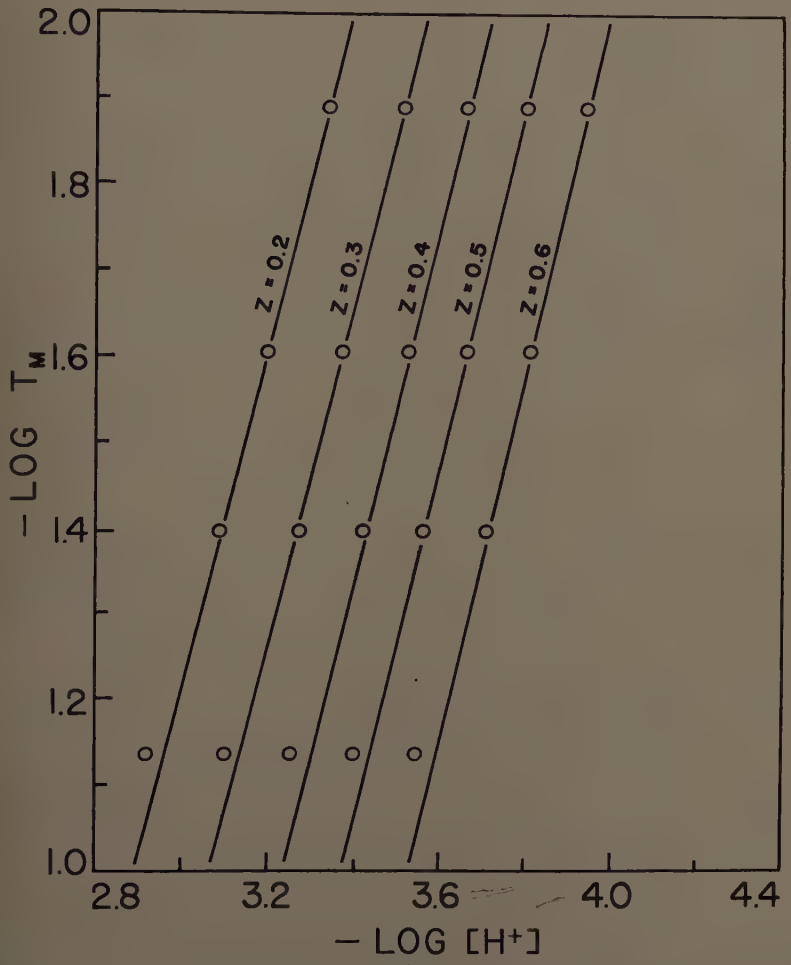


FIGURE 4. Plot of $-\log T_M$ versus $-\log [H^+]$ at constant Z based on data shown in FIGURE 3 for Fe(III)-HEDTA chelates.

fact that 1 mole of hydroxide ion is bound per mole of metal chelate is the dimer where n = 1. It should be pointed out that the presence of significant amounts of monohydroxo species in equilibrium with the dimer would tend to give values of $(\partial \log T_M/\partial \log [H^+])_Z$ in excess of 2.0. The fact that a value of 2.04 is obtained indicates that the dimer is by far the most predominant hydrolyzed species in solution in the concentration range studied.

In FIGURE 5, plots of $[H^+](T_{OH} + [H^+])/[MA]$ versus $2[MA]/[H^+]$ using the

same data plotted in FIGURES 3 and 4 show a definite drift toward higher intercept values as the concentration of metal chelate increases. In calculating the best straight line through the data, values obtained at the highest concentration (7.3×10^{-2} M) were not used since these data also did not conform

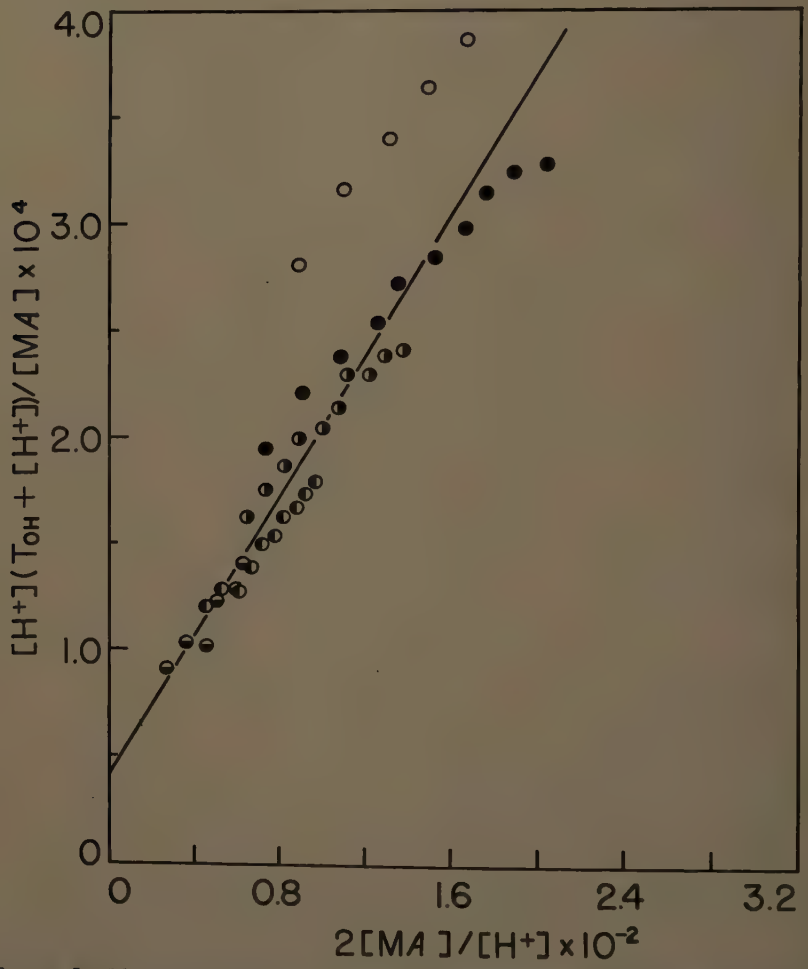


FIGURE 5. Plot of data of FIGURE 3 illustrating probable presence of the binuclear Fe(III)-HEDTA chelate. Concentrations: ○, 7.3×10^{-2} M; ●, 4.0×10^{-2} M; ◑, 2.5×10^{-2} M; ◐, 1.3×10^{-2} M; ◒, 6.6×10^{-3} M.

with the straight lines of FIGURE 4. No apparent explanation can be found for this inconsistency. It had been noted earlier that stock solutions of Fe(III)-HEDTA decomposed on standing, so that plots similar to those of FIGURE 5 yielded curves that nearly doubled back on themselves at high $[MA]/[H^+]$ values. This difficulty was eliminated by carrying out each titration with a freshly prepared chelate solution.

The constants obtained are shown in TABLE 3, where it may be seen that the tendency toward hydrolysis is considerably greater than in the case of the analogous Fe(III)-EDTA chelate. This suggests that binding takes place through the hydroxyethyl group and that an equilibrium of the type

$$Fe[OH]AOH^{1-} \rightleftharpoons FeAO^{1-} + H_2O$$

lies far to right. It is of course impossible to distinguish, on the basis of evidence now available, between the species $Fe[OH]AOH^{1-}$ and $FeAO^{1-}$.

Bogucki and Martell[7] have described the interaction of Th(IV) with HEDTA. Potentiometric titration curves show that after an initial inflection at $m = 3$ corresponding to dissociation of protons from the 2 nitrogen atoms and 1 acetic acid group, an additional $1\frac{2}{3}$ moles of base is consumed in producing a unique hydrolysis product. Plots of data obtained in the region $m = 3.0$–3.4 yield straight lines similar to that of FIGURE 2, showing that the initial reactions are similar to those indicated in equations **1** to **3**. The equilibrium constants obtained are shown in TABLE 3, where it may be seen that although the Fe(III) chelate hydrolyzes more readily, the Th(IV) chelate undergoes more extensive polymerization.

TABLE 3
HYDROLYSIS AND OLATION OF HEDTA CHELATES AT 25° C.

Metal	pK_a	pK_D	log K_d
Fe(III)*	4.4	5.8	3.0
Th(IV)†	5.4	5.6	5.2

* In 1.0 *M* KCl.
† In 0.1 *M* KNO_3. Probable product at *p*H 7: $ThA(Th[OH]_2A)_5$.

Ultracentrifugation of the Th(IV)-HEDTA polymer obtained at $m = 4\frac{2}{3}$ has shown that the product most probably is a hexamer. Bogucki and Martell[7] have shown, using potentiometric data in the range $m = 3$ to $4\frac{2}{3}$, that $(\partial \log T_M/\partial \log [H^+])_Z = 2.0$, suggesting that the empirical formula of the polymer is $ThAOH(Th[OH]_2AOH)_5$ or, preferably, a configuration in which the hydroxyethyl groups are bound to the thorium atoms.

The use of chelating agents containing phenolic groups often leads to the formation of polynuclear chelates of considerable complexity. Titration of equimolar mixtures of UO_2^{2+} and Tiron (pyrocatechol-3,5-disulfonate) results in a unique complex at *p*H 5.3 containing 2 hydroxo groups per 3 moles of metal ion.[8] The reactions and equilibrium constants involved are as follows:

$$UO_2 + H_2A \rightleftharpoons UO_2A + 2H^+$$

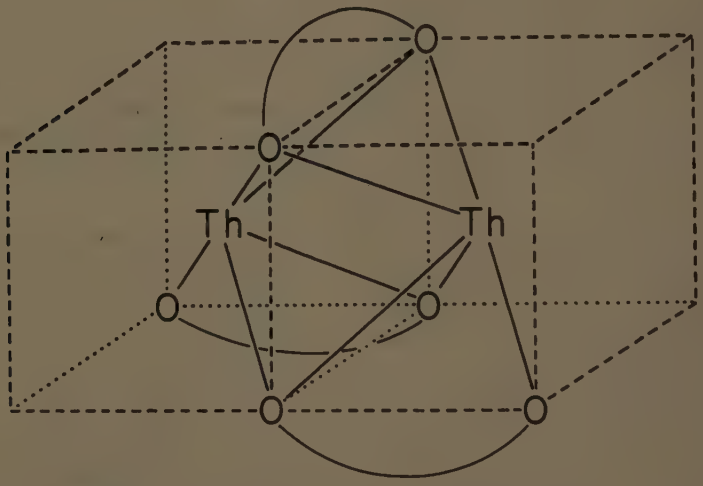
$$K_a = \frac{[UO_2A][H^+]^2}{[UO_2][H_2A]} = 10^{-4.13}$$

$$3UO_2A \rightleftharpoons UO_2A(UO_2[OH]A)_2 + 2H^+$$

$$K_b = \frac{[UO_2A(UO_2[OH]A)_2][H^+]^2}{[UO_2A]^3} = 10^{-2.8}$$

Although a dimeric form, $UO_2A[OH]UO_2A$, would appear to be the precursor of the trimer, inclusion of a term to account for the presence of such a species results in divergent values of the equilibrium constants, suggesting that the amount of dimer in equilibrium with monomer or trimer is insignificant.

Ultracentrifugation measurements[9] have shown that at *p*H 5 a dimeric chelate of composition Th_2Tiron_3 is formed wherein the bridging between the thorium atoms is accomplished by using the phenolate oxygen atoms. This is the first case found in these laboratories wherein bridging through hydroxo groups was not utilized. A possible structure of the chelate formed is shown in FIGURE 6. Although the structure has been drawn as though the donor groups about Th(IV) were at the corners of a cube, it is more probable that



THORIUM-TIRON (2:3) CHELATE

FIGURE 6. Diagram of possible configuration of Th_2 $Tiron_3$ chelate.

the configuration about each thorium atom is that of a square Archimedean antiprism. Solvent extraction experiments[10] utilizing Th^{234} tracer have verified the fact that the 1:1.5 Th(IV)-Tiron chelate exists as a dimer at *p*H 5.

The foregoing discussion has illustrated a few of the recent developments in the study of polynuclear metal chelates. In many cases chelated metal ions form only low molecular weight polymers, and the data pertaining to the formation of these compounds may be analyzed by relatively simple methematical treatments. In other cases, such as that of the hexamer of Th(IV)-HEDTA, the degree of complexity is such that up to the present time only the identification of the polymer formed has been achieved. In the future it is anticipated that the use of ultracentrifugation and solvent extraction techniques will be used to greater advantage in the elucidation of the degree of polymerization of chelates of highly charged metal ions.

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PARTICIPATION OF CHELATING METALS IN CARBOXYLATION REACTIONS

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Introduction

Chelate compounds, arising out of the combination of inorganic ions with polyfunctional organic molecules, may be expected to hold great interest for investigators in widely different fields of chemistry. The stability of many such substances can be an important factor both in the manipulation of ionic equilibria and in the alteration of organic structures. In the former application there has been great progress in recent years, so that the use of polyfunctional organic compounds as precipitants, titrants, and solubilizing agents for metals is now common. Alteration of the effective emf of a redox system such as represented in EQUATION 1, by addition of a chelating agent that will preferentially bind one of the metal ions (EQUATION 2), is likewise familiar.

$$M^n + [\mathrm{Ox}] \rightleftharpoons M^{n+1} + [\mathrm{Red}] \tag{1}$$

$$M^{n+1} + \mathrm{A\text{-}B} \rightleftharpoons \begin{bmatrix} A\text{-}B \\ \backslash\ / \\ M \end{bmatrix}^{n+1} \tag{2}$$

$$A + B \rightleftharpoons A\text{-}B \tag{3}$$

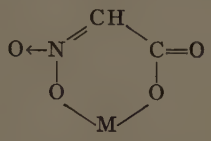
Use of analogous techniques to influence organic reactions has lagged far behind.[1] The importance of chelating metals in many of the organic reactions that occur in biological systems, though recognized, is poorly understood. Westheimer's clarification[2] of the catalytic effect of chelating metal ions upon the decarboxylation of oxaloacetic acid did not have synthetic utility, but it pointed to the potential importance of chelating metals in organic reactions in general. A very direct kind of influence which a chelating metal could have upon a reversible organic reaction is illustrated by EQUATIONS 2 and 3, where the formation of a stable chelate (EQUATION 2) serves to displace the equilibrium of EQUATION 3 in favor of the bifunctional product *A-B*. The improved yields in the preparation of certain chelating agents that result when copper ions are added to the reaction mixture[1] and the similar effect that borate recently has been reported[3] to have upon the formation of *p*-aminosalicylic acid may be examples of this "equilibrium effect" of chelation.

It was desirable to test the synthetic utility of this phenomenon in a more direct fashion by investigating a reversible reaction that otherwise fails, that is, one whose equilibrium position lies completely on the side of monofunctional compounds (*A* and *B* in EQUATION 3). The reaction chosen[4] was that of EQUATION 4. Steinkopf had shown[5] that nitroacetic acid decarboxylated completely,

$$\underset{\text{I}}{\mathrm{O_2N{-}CH_2{-}CO_2H}} \left(\overset{\rightarrow}{\underset{?}{\leftarrow}}\right) \mathrm{O_2N{-}CH_3} + \mathrm{CO_2} \tag{4}$$

and no evidence had appeared since then to suggest that the reaction ever proceeded in the opposite direction.

The evidence that nitroacetic acid (I) formed chelate salts was limited to Pedersen's observation[6] that certain metal ions (for example, Al^{+3}, Cu^{+2}, Mg^{+2}) retarded appreciably the rate of the decarboxylation reaction. It was found[4,7] that the aluminum and magnesium salts of I could be prepared in methanol or ether, and comparison of their spectra and stability characteristics with those of the alkali metal salts led to the proposal of the chelate structure (II). The magnesium salt (II, M = Mg) can be easily prepared by mixing equivalent



M = Mg or Al^{+}

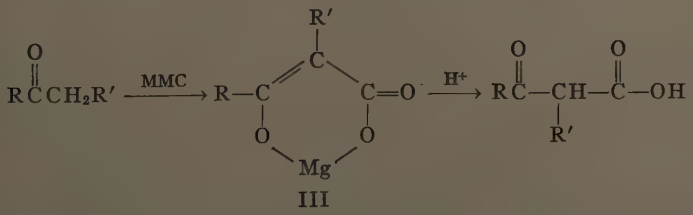
II

quantities of nitroacetic acid (I) and magnesium methoxide in methanol. The resulting solution is stable for hours at room temperature, in sharp contrast to solutions prepared in identical fashion from I and sodium methoxide.[7,8] Even the magnesium salt is quite sensitive to acid, however, being partially decarboxylated by even so weak an acid as carbon dioxide in methanol.[7]

Carboxylation with Magnesium Methyl Carbonate

Consideration of the properties of the chelate salts (II) made it possible to design experiments aimed at using chelation to reverse the reaction of EQUATION 4. This objective was accomplished in principle when the spectrum of II was observed to develop during experiments in which nitromethane was treated with magnesium methoxide (or a mixture of aluminum and sodium alkoxides) and carbon dioxide in methanol.[7] Careful hydrolysis of such solutions led to isolable quantities of nitroacetic acid (I). Further modification of the technique led to the development of magnesium methyl carbonate in dimethylformamide as a reagent for the carboxylation of primary nitroparaffins in yields that are satisfactory from a preparative point of view. Nitroparaffins that have been successfully carboxylated with this reagent include nitromethane,[4] nitroethane,[4] 1-nitropropane,[4] 1-nitrobutane,[4] 1-nitro-3-methylbutane (Stiles, unpublished experiments), and 3-β-nitroethylindole.[8]

Subsequently it was shown[9] that magnesium methyl carbonate (MMC) could be used to carboxylate ketones which contain enolizable methyl or methylene groups (EQUATION 5). The intermediate chelate salts of β-keto acids (III)



(5)

III

were characterized by their intense absorption in the ultraviolet and by the

fact that they could be alkylated and acylated at the α-carbon (see below). Ketones that have been carboxylated by this technique include acetophenone,[9] 1-tetralone,[9] 1-indanone,[9] cyclohexanone,[9] and cyclopentanone.[8]

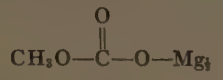
Role of Magnesium Ions in the Carboxylation Reactions

It is important to recognize that the success of the carboxylation technique just described rests on a kinetic effect exerted by the magnesium ions in addition to the simple equilibrium effect referred to earlier. The equilibrium of EQUATION 6 is completely to the left in all solvents studied[6,7]—water, methanol, and ether. One has clearly, therefore, to overcome this unfavorable equilibrium

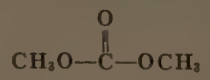
$$CH_2{=}NO_2^- + CO_2 \rightleftharpoons O_2NCH_2CO_2^- \qquad (6)$$

$$R{-}\overset{\displaystyle O^-}{\overset{|}{C}}{=}CH_2 + CO_2 \rightleftharpoons R{-}\overset{\displaystyle O}{\overset{\|}{C}}{-}CH_2CO_2^- \qquad (7)$$

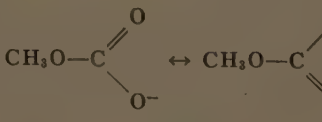
in the carboxylation reaction by conversion of the unstable monoanion to the chelate salt (II). This function of the metal ion may be designated the equilibrium effect. This same factor may be assumed to facilitate conversion of the enolate of a ketone to the β-keto acid salt (EQUATION 7) since there is good evidence that chelate salts (III) intervene in this system also. However, it must be recognized that the equilibrium of EQUATION 7 lies much further to the right, in an inert solvent, than that of EQUATION 6,* so much so that β-keto acids can be prepared in fair yields by treating an ether solution of the sodium or potassium enolate with carbon dioxide.[11,12] The equilibrium effect of magnesium ions is therefore not sufficient to explain the effectiveness of magnesium methyl carbonate in carboxylating ketones. The explanation of the additional kinetic effect appears to lie in the greater tendency of magnesium ions, compared to the alkali metals, to coordinate with the carboxyl groups of the reagent. The result is to make the carbonyl group of magnesium methyl carbonate (IV) resemble that of a carbonate ester (V) and hence susceptible to nucleophilic attack, in contrast to the inert carboxylate ion (VI).† Thus



IV



V



VI

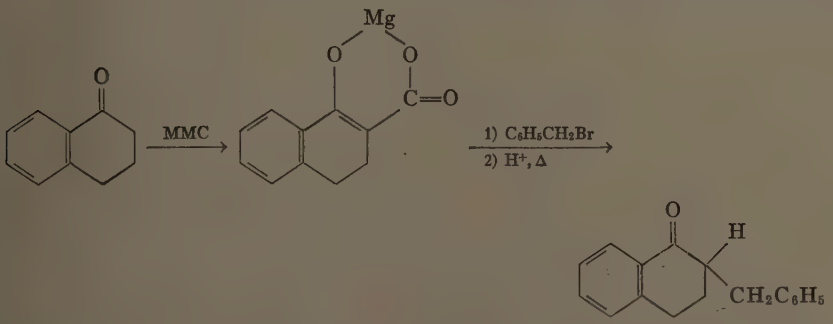
* In aqueous solution, bicarbonate ion and ketone or nitroparaffin are the stable species, so that decarboxylation is favored thermodynamically in both systems. However, in an aprotic solvent such as ether (and presumably in dimethylformamide) the relative positions of equilibrium are as stated above. It should be pointed out that the rates of decarboxylation in the two systems depend on acidity in opposite ways; the anion of an α-nitro acid is the reactive species,[6] while the free β-keto acid decarboxylates more rapidly than its alkali metal salt.[10] Nevertheless, both decarboxylations ultimately go to completion in aqueous solution regardless of whether the medium is acidic or alkaline.

† An alternative explanation of the kinetic effect is based upon the possibility that free carbon dioxide, present in the reagent, is the species which reacts with the enolate ion to

the presence of magnesium ions gives rise to a carboxylating species (IV) that is compatible with the basic catalyst (low concentrations of methoxide) necessary to generate the enolate ion from the ketone. Such a system can therefore be used as a one-step carboxylating reagent, and for this reason it is usually more satisfactory than the two-step method required in the absence of a chelating metal, particularly since the very strongest bases (sodium amide or triphenylmethide) must be used in the older technique.

Alkylation of Chelate Salts

The enolate structure assigned to the chelate salts (II and III) suggests that the substances should be reactive toward alkylating and acylating agents. This was found[9] to be the case for the β-keto acid derivatives (III). Addition of a reactive alkyl halide to the reaction product from a ketone and magnesium methyl carbonate leads to alkylation of the α-carbon atom. In some instances at least (EQUATION 8) the method is a practical synthetic procedure.[9] The success of this synthetic scheme tends to confirm the structure assigned to the



(8)

chelate salts. At the same time it emphasizes the way in which the magnesium salt of a β-keto acid can resemble a β-keto ester, a similarity discussed in the preceding section. Treatment of the chelate salts (III) with an acylating agent such as benzoyl chloride leads in similar fashion to attack at the α-carbon and ultimately to β-diketones (Stiles, unpublished experiments). However, the acylation reaction is complicated by the reaction between acid chlorides and the MMC reagent, so that it is not at present a useful synthetic procedure.

Treatment of the α-nitro acid salts (II) with ordinary alkyl halides has not resulted in the desired alkylation reaction. Attack of the halide in this case appears to occur at one of the oxygen atoms of the nitro group, leading to oxidation of the halide. Similar results have been reported in the attempted alkylation of nitroparaffin salts.[13] Use of gramine methiodide as the alkylating agent for the salt II has been successful, presumably because of the different mechanism by which this alkylating agent functions.[14] Thus nitromethane could be converted to the chelate (II), alkylated with gramine methiodide, and decarboxylated to furnish a high yield of 3-β-nitroethylindole.[7,8]

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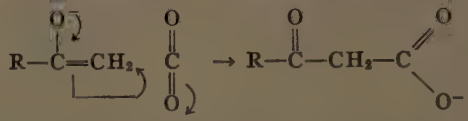
produce β-keto acid salt. Magnesium methyl carbonate solutions are known[7] to evolve CO_2 at much lower temperatures than sodium methyl carbonate, which is ineffective toward ketones. Current work is directed toward deciding this point.

Enzymatic Carboxylation Reactions

It is of interest to extend some aspects of the foregoing discussion to enzymatic carboxylation reactions. The reactions of direct interest will be those in which an "active hydrogen compound" such as a ketone or thiol ester, or a vinylog of such compound, is carboxylated at one of the enolizable positions. It is reasonable to assume that the actual bond-making process resembles, electronically, that of the nonenzymatic reaction, that is, it is a nucleophilic attack by an enol or enolate species upon the carbon atom of carbon dioxide or one of its derivatives, similar to the process of EQUATION 9. This representa-



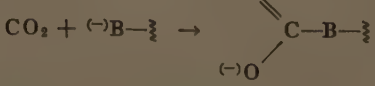
(9)

tion must be modified, however, to take into account the interaction of the reactants with the enzyme.

Although few precise data are available on the variation of reaction rates with carbon dioxide concentration in enzymatic reactions, there are at least qualitative indications[15-17] that the Michaelis-Menten law[18] is obeyed, and that CO_2 must be bound to the enzyme prior to its attachment to the substrate.* If one centers attention on the carbon dioxide reactant, it is therefore necessary to account for two events: (1) the binding of CO_2 (or a suitable derivative) to the enzyme, and (2) the transfer of the bound CO_2 to the substrate, which is assumed to be suitably activated and oriented by the enzyme. These two events can be unrelated chemically when the species under consideration is a large polyfunctional molecule, as is so frequently the case, but the small size and essentially monofunctional nature of carbon dioxide require that the two processes be closely connected. This connection can provide an insight into the nature of the binding, assuming only that the chemical behavior of CO_2 closely parallels that observed in nonenzymatic reactions.

The simplest mode of attachment of carbon dioxide would involve a single site that, in principle, could be either acidic (EQUATION 10) or basic (EQUATION

(10)



(11)

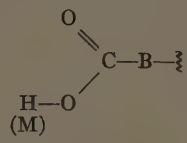
11). The former binding is very unlikely; nowhere in the chemistry of carbon

* The possibility that CO_2 might be incorporated into a coenzyme prior to interaction with the apoenzyme does not alter the argument that follows; it would then be the CO_2-coenzyme binding that concerned us. In those cases[16,19] where the saturation level of carbon dioxide is unusually high, one might suspect that an unfavorable equilibrium between carbon dioxide and some other substance present precedes the enzyme-catalyzed step. For example:

$$CO_2 + X \rightleftharpoons XCO_2$$

$$XCO_2 + \text{Enzyme} \rightleftharpoons \text{Complex}$$

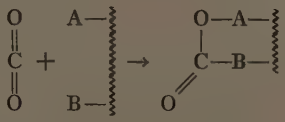
dioxide can one find evidence that the substance functions as a base.* The second possibility (EQUATION 11) is inherently very likely, for carbon dioxide combines energetically with bases of many varieties, but the resulting adduct cannot be expected to exhibit the required reactivity toward an enol or similar nucleophilic substrate. As pointed out in one of the earlier paragraphs, carboxylate ions show little tendency to enter into this type of addition reaction. The coordination of a proton or metal ion with the carboxylate ion to give a species such as VII (or the formation of an equivalent structure by combina-



VII

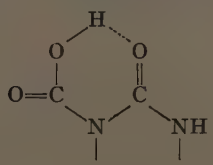
tion of bicarbonate with the enzyme) would lend the desired reactivity, but the acidity of VII (where B is oxygen or nitrogen) would be greater than that of acetate, for example, and hence the dissociation to the inert anion would be virtually complete in water at *p*H 7.

The foregoing argument leads to the conclusion that carbon dioxide needs to be attached to the enzyme at two sites (EQUATION 12) in order to be both bound and reactive.



VIII

The only experimental evidence to date on the nature of a CO_2-enzyme complex is that recently furnished by Lynen and his collaborators.[22] These investigators have shown that β-methylcrotonyl CoA carboxylase contains biotin units that are utilized in the catalytic action of the enzyme and, further, that the enzyme can be induced to catalyze the introduction of a carboxyl group onto one of the nitrogen atoms of free biotin. They have reasonably concluded that the biotin-CO_2 compound that they characterized is representative of the active carboxylating agent. Consideration of the detailed structure of biotin-CO_2 (partial structure IX), including the indubious chelate ring, shows

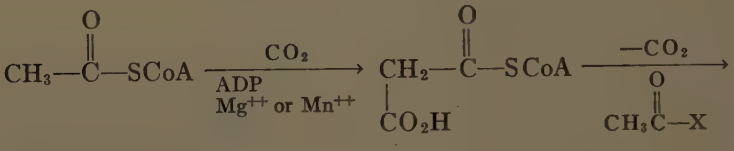


IX

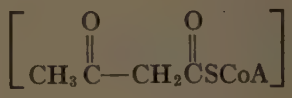
* The solubility of carbon dioxide in sulfuric acid is approximately the same as in water; less, in fact, than in various hydrocarbon solvents.[20,21] Experiments designed to discover specific interactions between CO_2 and BF_3 in the gas phase gave no evidence for such interaction (R. S. Berry, private communication).

that it fulfills the requirements outlined above for reactive carbon dioxide. Although no requirement for a chelating metal is associated with the function of Lynen's enzyme-biotin-CO_2 complex,[22] the general need for such metals in enzymatic carboxylations of varied type[23] suggests that instances may be found in which a metal ion fulfills the role played by the chelated hydrogen in IX (that is, A in formula VIII).

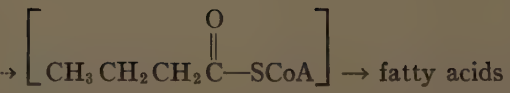
Recent work on the enzymatic synthesis of fatty acids[15,24,25] has revealed a pattern of steps in which carboxylation of an "active hydrogen" compound is followed by such reactions as acylation, decarboxylation, and reduction, as summarized in EQUATION 13. The similarity of this sequence to the pattern



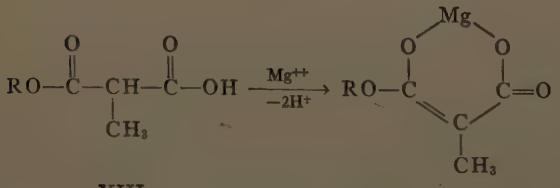
X XI (13)



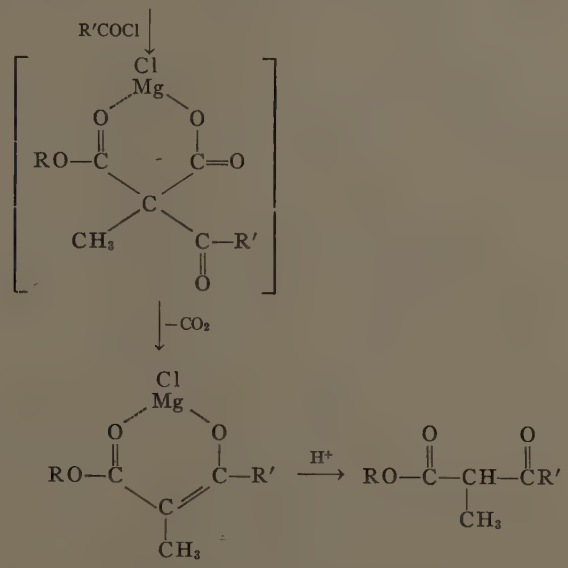
XII



of acylation and alkylation reactions that has been observed to involve chelate salts (EQUATION 8, for example) is striking. The involvement of malonyl CoA (XI) in chelation would provide an attractive rationale for its intervention in this biosynthetic scheme. In fact, a very recent laboratory synthesis for substituted β-keto esters furnishes a precise model for this series of transformations. Ireland and Marshall[26] have found that monoesters of malonic acids (XIII) can be converted to chelate salts XIV, which are readily acylated and decarboxylated to give the β-keto ester product XV.



XIII XIV



XV

It is obvious that the occurrence of chelation phenomena in organic reactions goes far beyond the examples that have been discussed here. One can expect to find that in the future, chelation will find increasing use in the deliberate control of organic transformations. A thorough understanding of the importance of chelation in biological systems, even in the limited field of carboxylation reactions, must await more precise information from studies of the chemical effects of chelation, as well as from studies of intermediate structures in enzyme reactions.

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THE EFFECT OF STRUCTURAL MODIFICATIONS ON POLYAMINEACETIC ACID CHELATING AGENTS*

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The polyamine acetic acids, of which ethylenediaminetetraacetic acid (EDTA) is the best known member, represent a class of chelating agents finding widespread applications in biology and chemistry. The effective use of these compounds requires a knowledge of their metal-binding properties in the specific environment in which they are to be used. Frequently, the choice of a chelating agent can exert a profound effect on the quantitative aspects of an experiment. To cite several examples, diethylenetriaminepentaacetic acid (DTPA) is the most effective of the polyamine acetic acids in removing radioactive rare earths and plutonium from both humans and animals.[1,2] Bis-[(dicarboxymethyl)aminoethyl] ether has a pronounced effect in increasing the urinary excretion[3] and decreasing the deposition of radiostrontium in bones[4] of rats exposed to the radioisotope. Cyclohexane *trans*-1,2-diaminetetraacetic acid (CDTA) is more effective than EDTA in inhibiting the hemolysis of red cells acted on by a specific antibody-complement complex (P. Lalezari, private communication, 1959). Ethylenediamine bis(*o*-hydroxyphenyl acetic acid)[5] is the compound of choice in supplying iron to plants grown in alkaline media or soils and in investigating iron metabolism in humans (*see* S. Korman, elsewhere in this monograph).

The judicious use of a chelating agent requires a knowledge of the stabilities of its metal chelates as measured by the stability constant. This is a function of a complex group of parameters dependent on the properties of the metal ion and the structural chemistry of the organic chelating agent.[6] The chelation reaction in aqueous solutions consists of the displacement of solvated water surrounding the metal cation by the metal-bonding atoms of the organic molecule,[7] and the capacity of these electronegative atoms to assume the optimal symmetry required for stable metal chelate ring formation is dependent on the structure of the organic molecule.

The equilibriums of alkaline earth cations with chelating agents are ideal systems for evaluating the structural contributions of the organic molecule to the chelation reaction. The alkaline earth cations of magnesium, calcium, strontium, and barium have a regular increase in ionic radii while maintaining a constant valency of 2. The bonds between metal ion and ligand atoms are ionic, and the multidentate chelates have a tetrahedral or octahedral configuration.[8]

FIGURE 1 shows plots of the logarithms of the stability constants (log K) of a number of alkaline earth polyamine acetate chelates. These values are arranged in the order of increasing ionic radii of the cations. The chelating agents in these graphs are grouped on the basis of the following structural similarities:

* The work described in this paper was supported in part by the United States Atomic Energy Commission, Contract AT(30-1)2094, Washington, D.C.

Carbocyclic (cyclohexane and cyclopentane) *trans*-1,2-diaminetetraacetic acids (CDTA and CPDTA), with EDTA included for comparison (FIGURE 1*a*).

Alkylenediaminetetraacetic acids containing one central ligand atom or group having the general formula $(HOOCCH_2)_2NCH_2CH_2XCH_2CH_2N(CH_2 \cdot COOH)_2$, where X = —$NCH_2COOH$, O, CH_3N—, S (FIGURE 1*b*).

Alkylenediaminetetraacetic acids containing two central ligand atoms or

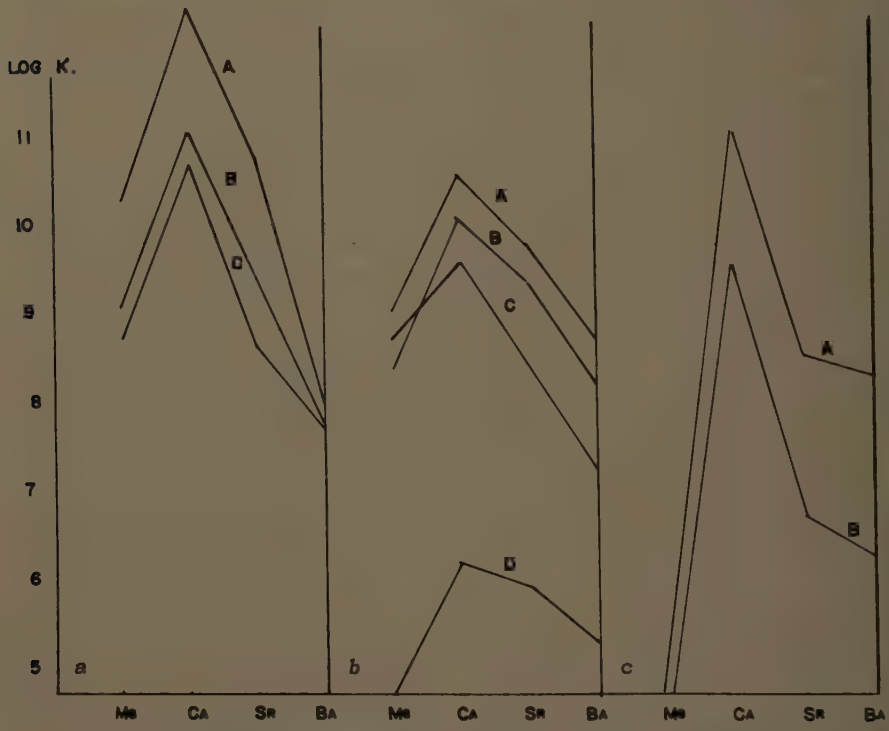


FIGURE 1. Logarithms of the stability constants. (*a*) *A* = cyclohexane *trans*-1,2-diaminetetraacetic acid; *B* = cyclopentane *trans*-1,2-diaminetetraacetic acid; *C* = ethylenediaminetetraacetic acid. (*b*) *A* = diethylenetriaminepentaacetic acid; *B* = bis[(dicarboxymethyl)aminoethyl] ether; *C* = bis[(dicarboxymethyl)aminoethyl] methylamine; *D* = bis[(dicarboxymethyl)aminoethyl] sulfide. (*c*) *A* = bis[(dicarboxymethyl)aminoethoxy] ethane; *B* = bis[(dicarboxymethyl)aminoethyl] methylamine (BDAM).

groups having the general formula $(HOOCCH_2)_2NCH_2CH_2XCH_2CH_2XCH_2 \cdot CH_2N(CH_2COOH)_2$, where X = O, CH_3N— (FIGURE 1*c*).

It is interesting to note that the slopes of the curves in each group are similar. The common feature of the three groups is that the metal chelate stability sequence is Mg < Ca > Sr > Ba. However, each of the three groups of compounds exhibits quantitative differences with respect to this stability sequence.

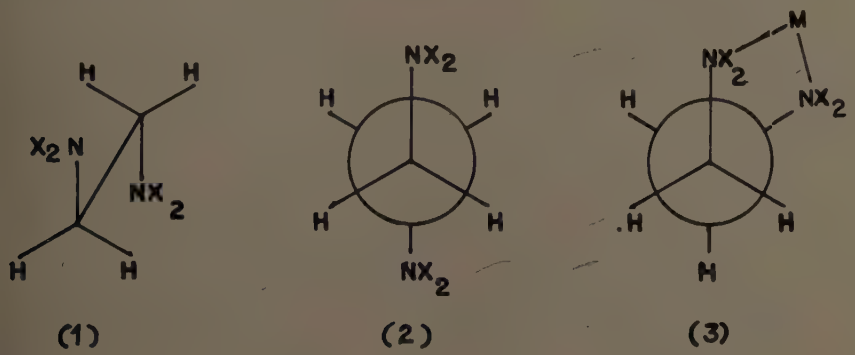
In FIGURE 1*a* the contribution of the hydrocarbon skeleton to the metal chelate stability is cyclohexane[9] (*A*) > cyclopentane[10] (*B*) > ethane[7] (*C*). This effect may be attributed to the stereochemistry of the carbocyclic rings and, possibly, to their dielectric contribution to the chelation reaction. This

subject will be discussed in a subsequent section. Furthermore, this group of compounds is characterized by a high stability of the calcium chelate relative to magnesium, strontium, and barium.

The compounds in FIGURE 1*b* contain a central metal-binding group, whose contribution to the alkaline earth chelate stability[7,11] is $NCH_2COOHA > OB > CH_3N—C > SD$. A property of these compounds is that the stabilities of the calcium and strontium chelates are closer together than in the other two classes of polyamine acetic acids. This difference in calcium and strontium stabilities ranges from a factor of 10 for DTPA to 2 for bis[(dicarboxymethyl)-aminoethyl] sulfide. These values are to be compared with the hundredfold difference in chelate stability of EDTA for the same metal ions. Chelating agents belonging to this group of compounds are under investigation for removing radiostrontium from experimental animals.

The two compounds in FIGURE 1*c* corroborate in part the conclusion drawn from the previous set of chelating agents. The contribution of the two central metal-binding groups to the chelate stability is $OA > CH_3NB$. The unique features of these two compounds are that (1) the calcium chelate stability constant is approximately 10^6 times greater than that of the magnesium and (2) the strontium and barium chelate stabilities are relatively close together.

Schwarzenbach has described the chelation reaction as a stepwise process in which the metal cation first is bound by a ligand atom of a polyfunctional chelating agent.[6] The internal rotation of the molecule permits a neighboring ligand atom to move to a second coordination site on the central ion to complete the chelate ring. This rotation is illustrated in FIGURE 2 by the perspective formula (formula 1) and Newman projection formulas[12] (formulas 2 and 3) of EDTA.



where X = —CH_2COOH.

The ethylenediaminetetraacetate anion in aqueous solution has a *trans* orientation with respect to the negatively charged iminodiacetate groups (formula 2). The bonding of the metal by the end ligand group must be followed by an internal rotation about the ethane carbon—carbon bond to yield the configuration in formula 3. In going from the conformation of formula 2 to that of 3, the molecule must pass through an energy barrier that is attributed to the repulsive interaction of the substituents on the carbon atoms as they eclipse one another.[13]

In CDTA and CPDTA the free rotation of the carbon—carbon bond associated with the diamine structure is limited, and therefore the nitrogens of the iminodiacetate groups are spatially fixed. A comparisoe of the chelation of alkaline earths by CDTA (formula 4) and CPDTA (formula 5) permits an evaluation of the effect of this restricted rotation on metal chelate stability.

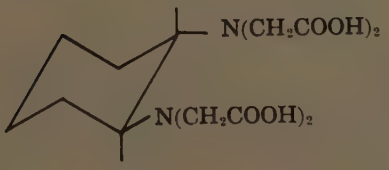
The iminodiacetic acid substituents in CDTA occupy equatorial positions on the cyclohexane ring, as indicated by the large difference between the pK_a of the first two acid dissociation constants in formula 4[14] (*see also* TABLE 1). It is likely that the ionized iminodiacetate groups are diaxial because of the electrostatic repulsion of their negative charges[15] (formula 5). Chelation with a metal ion changes the conformation of the cyclohexane ring so that these groups

TABLE 1
STABILITY CONSTANTS OF THE ALKALINE EARTH CHELATES OF CDTA[9] AND CPDTA*[10]

	CDTA	ΔpK	CPDTA	ΔpK
pK_1	2.43		2.41	
		1.09		0.51
pK_2	3.52		2.92	
pK_3	6.12		7.42	
		5.58		2.89
pK_4	11.70		10.31	
		log K		Δ log K
Mg	10.32		9.05	1.27
Ca	12.50		11.08	1.42
Sr	10.69†		9.45	1.24
Ba	7.99		7.75	0.24

* T = 20° C.; μ = 0.1.
† Kroll, unpublished data.

occupy equatorial positions that facilitate the formation of the chelate ring (formula 6).



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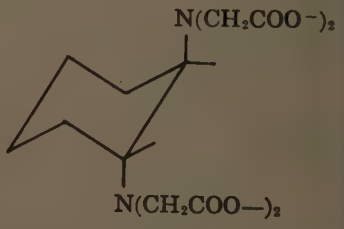

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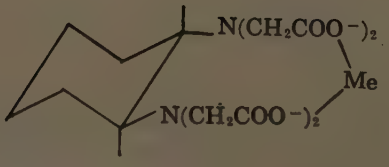
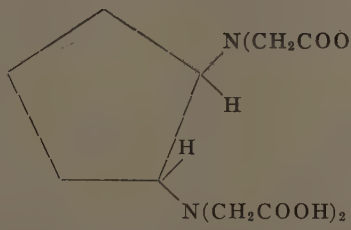


(6)

The nitrogens in the diaxial position are separated by approximately 3.4 Å, and in the conversion to the diequatorial conformation this separation is decreased to 2.2 Å. The nitrogens in CPDTA (formula 7) can be considered as fixed in space. The effect of the puckering of the cyclopentane ring on the chelating groups is disregarded.[16] In this molecule, the nitrogens are estimated as separated by 2.6 Å.

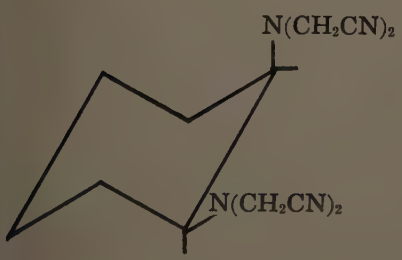


(7)

The stability constants of the alkaline earth chelates of CDTA and CPDTA are shown in TABLE 1.

The larger ΔpK values for CDTA over those of CPDTA (TABLE 1) support the conclusion that the carboxyl and nitrogen groups of the former are close together and therefore influence their respective acid dissociation processes. The magnesium, calcium, and strontium stability constants for CDTA are 20 to 30 times greater than the corresponding CPDTA values ($\Delta \log K$). The differences in stabilities between these two chelating agents for the alkaline earth group are attributed to the spatial arrangements of the ligand atoms in the chelate rings. These structures will depend on the positions of the two amino nitrogens relative to one another in the cyclohexane and cyclopentane derivatives.

The nitrogens of *cis*-1,2-diamine cyclohexane occupy axial and equatorial positions, and are separated by approximately 2.0 Å. The diamine has been converted to the cyclohexane *cis*-1,2-diaminetetraacetic acid (formula 9) via



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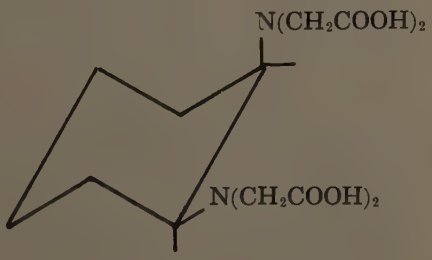
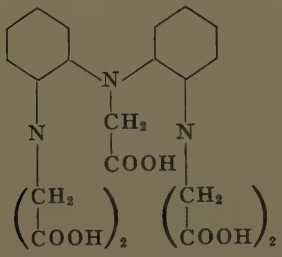


(9)

the cyanomethylated intermediate (formula 8) (Kroll and Gordon, unpublished data). Although quantitative chelation studies of the compound given by formula 9 have not been completed, potentiometric studies have shown that it is a strong chelating agent. Two properties that distinguish it from its *trans* isomer is its greater solubility in water and the greater acidity of the first ammonium nitrogen of the diamine moiety ($pK_{3(cis)} = 5.6$; $pK_{3(trans)} =$

6.1). This acidity of the *cis* derivative may be attributed to the closer proximity of the diamine nitrogens.

The substitution of the cyclohexane ring for the ethane carbon skeleton in two polyamine acetic acids failed to enhance the metal chelate stabilities of these compounds. Bis[(dicarboxymethyl)2-aminocyclohexyl] glycine[17] (formula 10) was found to be a weaker chelating agent than DTPA (formula 11), whereas bis[(dicarboxymethyl)2-aminocyclohexyl] sulfide[18] (formula 12) had approximately the same avidity for alkaline earths as did its aliphatic analogue (formula 13).



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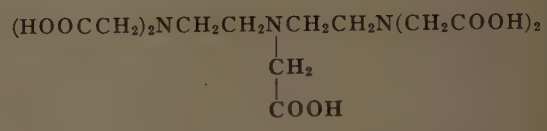

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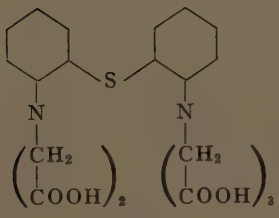

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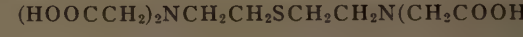
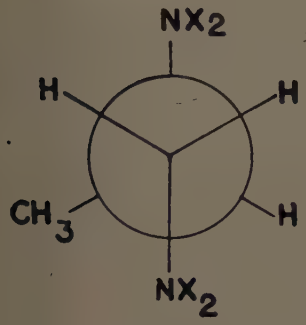


(13)

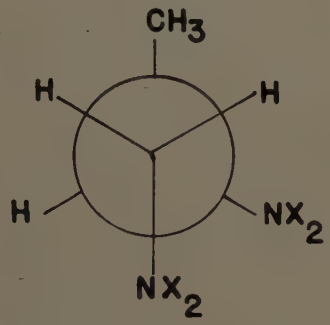
It has been indicated that EDTA has a *trans* configuration in aqueous solution and, in the chelation reaction, a rotation about the ethane carbon-carbon bond places the iminodiacetate groups in favorable positions for metal chelate ring formation. The rotation is opposed by the substituents on the carbon atoms as they eclipse each other and, persumably, the energy required to overcome this opposition to metal chelate formation is manifested in the stability constant. Replacing one of the hydrogens in the ethane chain of EDTA by a methyl group to yield propylene 1,2-diaminetetraacetic acid (PDTA), permits an evaluation of the steric effect of the alkyl group on the chelation reaction. The Newman projection formulas of PDTA are indicated in formulas 14 and 15 (on page 347) where X = —CH_2COOH.

If the two nitrogens are to participate in chelate ring formation, the methyl group must eclipse a hydrogen to assume a position midway between the two hydrogens on the adjacent carbon atom (formula 15). Conversely, once the optimal configuration for chelate formation has been attained the same energy barrier will oppose the reverse reaction to yield the *trans* species. Furthermore, since the metal ions involved in chelate ring formation have a considerable variation in ionic radii, the accommodation of the metal into a strain-free five-membered chelate ring will require some movement of the diamine nitrogens. This adjustment of position by the nitrogen atoms is accomplished by

rotation of the ethane carbon—carbon bond, which in turn is influenced by the substituents on these two carbon atoms. This steric effect should be reflected in the stabilities of the metal chelates of PDTA. Table 2 lists the stability constants for some divalent metal ions, and for EDTA and PDTA.[19] The table makes several interesting disclosures. The strontium chelate of PDTA is claimed to be one-hundredfold more stable than that of strontium EDTA. Further, strontium PDTA is somewhat more stable than the corresponding



(14)



(15)

Table 2
Metal Chelate Stability Constants of EDTA[7] and PDTA*[19]

	PDTA	EDTA
Ba	8.1	7.76
Ca	10.4	10.70
Cd	16.0	16.59
Co	14.4	16.21
Cu	17.7	18.79
Fe	14.3	14.3
Hg	21.7	21.8
Mn	14.5	13.58
Ni	14.2	18.56
Pb	17.3	18.3
Sr	10.7	8.63
Zn	16.2	16.26

* $T = 20°$ C.; $\mu = 0.1$.

calcium chelate. This is the only known reported stability constant indicating a reversal in the calcium-strontium sequence in the polyamine acetic acid series. The compound is being tested for the removal of radiostrontium in rats, although Catsch[4] has already shown that it is not as effective as the sodium salt of bis[(dicarboxymethyl)aminoethyl] ether in preventing the deposition of Sr^{90} in bones of rats. The differences in stability between the EDTA and PDTA chelates of cobalt, manganese, and nickel, as well as strontium and barium are of considerable interest, but it is premature to interpret these data in terms of specific steric effects of the methyl group on chelation reaction.

Other methyl-substituted polyamineacetic acids are being studied. Bis-

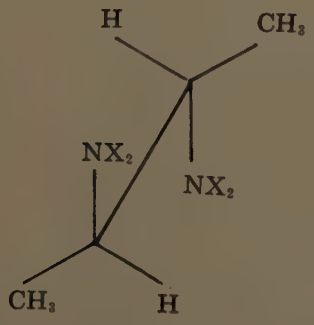
[(dicarboxymethyl)2-aminopropyl] ether (formula 16), bis[(dicarboxymethyl)2-aminopropyl] sulfide (formula 17), and dipropylenepentaacetic acid (formula 18) have been prepared.[20]

(16, 17, 18)

where X = O, S, —NCH_2COOH.

The ether (formula 16) and thioether (formula 17) have approximately the same avidity for earth cations as do the nonsubstituted analogues. Dipropylenediaminepentaacetic acid has a lower affinity for the alkaline earths than does DTPA.

A recent report from England states that butylene 2,3-diaminetetraacetic acid has strong metal-binding properties (H. Irving, personal communication, 1959). This molecule may exist in both a *meso* (formula 29) and DL (formula 20) configuration, and a comparison of their metal chelate stability constants should contribute to an understanding of the stereochemistry of the chelation reaction:



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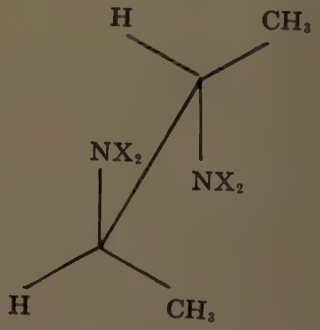


(20)

where X = —CH_2COOH.

To summarize the effect of methyl substituents on the chelating properties of the polyamine acetic acids: there is qualitative evidence that the methyl group does influence metal chelation when substituted on the carbon skeleton bonding the iminodiacetic acid moieties. The quantitative interpretation must await the availability of stability constant measurements and other physical chemical data.

The stabilities of the polyamine acetic acid chelates are enhanced by increasing the number of carboxylic acid groups in the molecule. TABLE 3 lists the alkaline earth stability constants of DTPA (formula 12)[11] and bis[(dicarboxymethyl)aminoethyl] methylamine (BDAM),[7] shown in formula 21.

$$\underset{\displaystyle\qquad\qquad\qquad\qquad\qquad\qquad\quad | \atop \qquad\qquad\qquad\qquad\qquad\qquad\quad X}{(HOOCCH_2)_2NCH_2CH_2NCH_2CH_2N(CH_2COOH)} \qquad (12, 21)$$

where X = —CH_2COOH (DTPA) or CH_3N(BDAM).

The replacement of the methyl group in BDAM by a carboxymethyl group (DTPA) increases the stability of the metal chelate by factors ranging from 10 to 70 (Δlog K). The largest increase in stability is for the magnesium ion.

A similar enhancement of stability has been observed in a modification of (dicarboxymethyl)aminoethyl sulfide (formula 13). The stability of the strontium chelate of this polyamine acetic acid approaches that of the calcium complex, and it is of interest in the removal of radiostrontium from experimental animals. However, the titration curves of formula 13 show that only about 15 per cent of the calcium and strontium chelates are formed at *p*H 7.3 to 7.5. The instability of these chelates in this *p*H range eliminated the use of the chelating agent in the removal of radiostrontium from experimental animals.

Table 3

Alkaline Earth Stability Constants of DTPA and BDAM*

	DTPA	BDAM	Δ log K
Mg	9.03	7.31	1.72
Ca	10.63	9.60	1.03
Sr	9.68	8.35	1.33
Ba	8.63	7.21	1.42

* $T = 20°$ C.; $\mu = 0.1$.

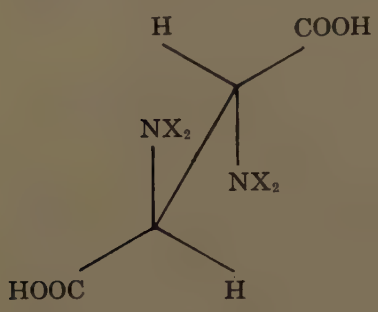
For purposes of increasing the stabilities of the thioether polyamine acetic acid chelate, a carboxylated analogue was synthesized. Lanthionine was treated with sodium bromoacetate to yield N,N,N′,N′-tetrakis(carboxymethyl) lanthionine (formula 22).[20] The lanthionine used was a mixture of the *meso* and DL forms.

$$(HOOCCH_2)_2N\underset{\displaystyle COOH}{CH}CH_2SCH_2\underset{\displaystyle COOH}{CH}N(CH_2COOH)_2 \qquad (22)$$

The compound differs from bis(dicarboxymethyl)aminoethyl sulfide (formula 13) in having carboxyl substituents on the carbons alpha to the iminodiacetate nitrogens. The titration curves for the absence and presence of calcium and strontium indicate a greatly increased stability of the chelates of calcium and strontium.

Recent studies in our laboratory have shown that DL-N,N,N′,N′-tetrakis-(carboxymethyl)1,2-diaminosuccinic acid (formula 24) is a strong chelating agent, whereas the *meso* isomer has a considerably reduced affinity for the alkaline earths. This observation is in agreement with the stereochemical require-

ments for metal chelation, since only the DL form (formula 24) can assume the proper configuration for metal chelate formation.



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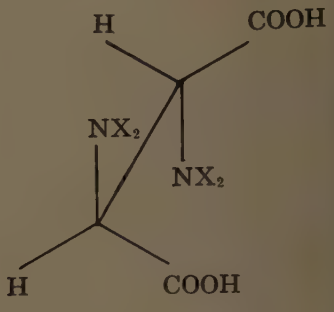


(24)

where X = —CH_2COOH.

TABLE 4

ALKALINE EARTH STABILITY CONSTANTS FOR SEVERAL POLYAMINE ACETIC ACID CHELATING AGENTS CONTAINING HYDROXY SUBSTITUENTS*

	HTDTA	TDTA
Mg	4.35	6.02
Ca	6.18	7.12
Sr	5.58	5.18
Ba	3.99	4.24

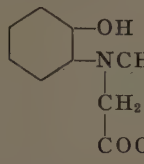
	HEDTA	HCEDTA	CEDTA
Mg	5.78	5.83	
Ca	8.14	8.39	1.3†
Sr	6.92	6.14	
Ba	5.54	4.68	

* T = 20° C.; μ = 0.1.
† T = 24° C.; μ = ca. 0.16.

The stability constants of the alkaline earth chelates of three hydroxy-substituted polyamine acetic acids were determined.[20] The compounds studied were 2-hydroxy 1,3-propylenediaminetetraacetic acid (formula 25), hydroxyethyl ethylenediaminetriacetic acid (formula 26), and 2-hydroxycyclohexyl ethylenediaminetriacetic acid (formula 27). The stability constants are given in TABLE 4.

$$(HOOCCH_2)_2NCH_2\underset{\displaystyle OH}{\underset{|}{C}}HCH_2N(CH_2COOH)_2 \quad \text{(HTDTA)} \tag{25}$$

$$\begin{matrix} HOCH_2CH_2 \\ \qquad\quad\diagdown \\ \qquad\quad\ NCH_2CH_2N(CH_2COOH)_2 \\ \qquad\quad\diagup \\ HOOCCH_2 \end{matrix} \quad \text{(HEDTA)} \tag{26}$$



(HCEDTA) (27)

$(HOOCCH_2)_2NCH_2CH_2CH_2N(CH_2COOH)_2$ (TDTA) (28)

The values for 1,3-trimethylenediaminetetraacetic acid (formula 19) and *N*-cyclohexyl ethylenediamine triacetic acid are included for comparison.

The introduction of a hydroxy group on the middle carbon atom of trimethylenediaminetetraacetic acid (TDTA) (formula 28) decreases markedly the stability of the calcium and magnesium complexes. However, this trend is reversed for the strontium ion. There is a hundredfold difference between the stability constants of the calcium and strontium chelates of TDTA, whereas the difference in the corresponding chelates of HTDTA is only about fivefold, and the strontium chelate of HTDTA is actually more stable than the strontium TDTA. This effect of hydroxy substitution on strontium chelation holds some promise for developing more selective chelating agents for this cation.

The replacement of the 2-hydroxyethyl group in HEDTA by the 2-hydroxycyclohexyl group in the group in HCEDTA has little effect on the chelation of calcium and magnesium. The HEDTA forms significantly more stable chelates with strontium and barium than does the cyclohexyl analogue. The stability constant of cyclohexylethylenediaminetriacetic acid (CEDTA) has been included in this table to illustrate the potentiating effect of the hydroxy group on metal chelate formation. Although the participation of the hydroxy group cannot be detected in the chelation of the alkaline earth ions by the usual potentiometric measurements, its involvement in the reaction must be accepted on the basis of the large difference that exists between the calcium chelate stability constant of HCEDTA and that of CEDTA. The coordination process binds the hydroxy group without displacement of its proton.

Summary

Quantitative and qualitative data are presented on the structural chemistry of polyamine acetic acids and their chelation of alkaline earth cations. The nature and arrangement of the ligand atoms in the organic molecule and its structural chemistry exert a considerable influence on the chelation of a metal ion. Although no exclusive cation specificity can be attributed to any single chelating agent, quantitative differences in the stabilities of metal chelates do exist, and these differences may be utilized in biological and chemical applications.

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Part II. The Biological Significance of Chelation

THE COPPER PROTEIN, ASCORBIC ACID OXIDASE

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Ascorbic acid oxidase, an enzyme that catalyzes the aerobic oxidation of L-ascorbic acid, has been extensively investigated since it was first described as hexoxidase by Szent-Gyorgyi in 1930.[1] When a critical role for copper in the enzyme's activity became apparent about ten years later, much additional interest in the enzyme was stimulated.[2,3] At that time it had long been known that copper salts markedly catalyze the aerobic oxidation of aqueous solutions of the antiscorbutic vitamin.[4] Consequently, the existence of a specific copper-protein enzyme was viewed with considerable skepticism by some workers in the field, notably Lampitt and Clayson[5] and Lampitt *et al.*[6]

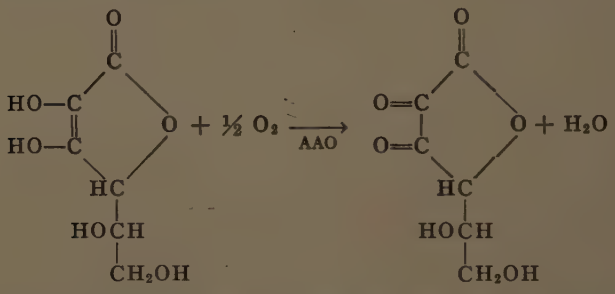
In this connection it is now worth noting that the enzyme-catalyzed oxidation of L-ascorbic acid exhibits a different oxygen stoichiometry than does the cupric ion-catalyzed reaction (FIGURE 1). In the latter case twice as much oxygen is absorbed, and hydrogen peroxide is readily detected as a terminal product along with the dehydroascorbic acid. In the enzyme reaction no hydrogen peroxide can be detected. Furthermore, when these two systems are compared on an equivalent copper basis, the enzyme copper is found to be about one thousand times more effective as a catalyst and exhibits a substrate specificity not observed with the ionic copper. For example, the enzyme catalyzes the aerobic oxidation of L-ascorbic acid much more rapidly than D-ascorbic acid.[7] The cupric ion catalysis does not exhibit this specificity, being equally effective for both D- and L-ascorbic acid. These observations, and many others made during the isolation and purification of the enzyme from a variety of plant sources, have convincingly established the existence of ascorbic acid oxidase as a specific protein-copper complex.[8] The copper is nondialyzable at physiological *p*H, and is not removed by an ion-exchange resin. No exchange of copper occurs when the highly purified enzyme is treated with ionic Cu^{++} labeled with radioactive Cu^{64}. These observations reveal that the copper in the enzyme protein-copper complex is very tightly bound, and cannot be the result of simple adsorption of copper to nonspecific protein matter.

The properties of ascorbic acid oxidase are those of a globular protein having a molecular weight of 150,000 and containing about 0.26 per cent copper, corresponding to 6 copper atoms per molecule.[9] Additional information about the purified enzyme is summarized in TABLE 1. Although the enzyme has never been crystallized, concentrated solutions of homogeneous protein, as adjudged by ultracentrifugation and electrophoreses, have been obtained and found to have a specific activity of about 2000 units per milligram of protein. This specific activity has served as a basis for estimating the degree of purity of other preparations. The pure enzyme is insoluble in pure water and can be precipitated from dilute salt solutions by dialysis against water. This property is useful in the final stages of purification.

The rind of the summer crookneck squash (*Curcurbita pepo condensa*) is one of the richest sources of the enzyme but, as indicated in FIGURE 2, the preparation of pure enzyme is a laborious process involving many steps. These steps, involving such factors as fractional salt precipitations and fractional adsorptions,[10] produce the pure enzyme in about 10 per cent yield with a purification factor of about 1300, that is, 1.5 units/mg. in the crude extract to 2000 units/mg. specific activity in the purified enzyme.

Dilute acetate buffer (0.1 *M*) solutions of the pure enzyme, containing more than 1 mg. of enzyme protein per milliliter, have a distinct blue to blue-green color. The intensity of this blue color to the eye, on an equivalent copper basis, is much greater than that of aqueous $CuSO_4$ or solutions of the copper ammonia

ENZYME-CATALYZED OXIDATION OF L-ASCORBIC ACID



CUPRIC ION-CATALYZED OXIDATION OF L-ASCORBIC ACID

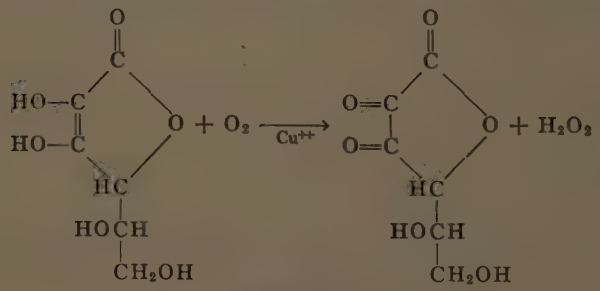


FIGURE 1.

complex. Thus a solution of the pure oxidase containing 60 γ Cu/ml. has about the same intensity of blue color as a $CuSO_4$ solution containing 40,000 γ Cu/ml., or between 500 and 1000 γ Cu/ml. of the $Cu(NH_3)_4^{++}$ complex.

When a small amount of ascorbic acid is added to a blue solution of ascorbic acid oxidase in 0.1 *M* acetate buffer at *p*H 5.6, the color is rapidly bleached to a light yellow (FIGURE 3). Then, as oxygen is admitted to the system, the blue color returns relatively slowly. This process of bleaching and recolorizing with ascorbic acid and oxygen can be repeated several times.[8]

The data in FIGURE 4 show how the phenomenon manifests itself spectrophotometrically (D. M. Kirschenbaum and C. R. Dawson; to be published). The visible absorption curve of the blue enzyme is characterized by a distinct peak at 606 μ, a small shoulder at 412.5 μ, and a minimum in the neighborhood

of 500 μ (Curve 1). It is to be noted that when ascorbic acid is added (Curve 2), the 606-μ peak disappears, but the shoulder at 412.5 μ is not affected.

Before giving consideration to the problem of attempting to identify the types of structures or groups involved in chelating the copper to this particular protein, certain additional facts about the stability of this copper protein bond should be presented. First: although the activity and copper content of con-

TABLE 1
ASCORBIC ACID OXIDASE[9]

Source: summer crookneck squash (*Curcurbita pepo condensa*)
Preparation: differential ultracentrifugation and fractional precipitation by dialysis
Specific activity: 2000 U./mg.
Color: blue-green (dependent on O_2 and substrate)
Homogeneity: 100% (ultracentrifuge and electrophoresis)
Molecular wt.: 150,000
Copper: 0.26%, corresponding to 6 Cu atoms per mole
Activity per Cu: 740 U.
Nitrogen: 16.8%, no P or Ca

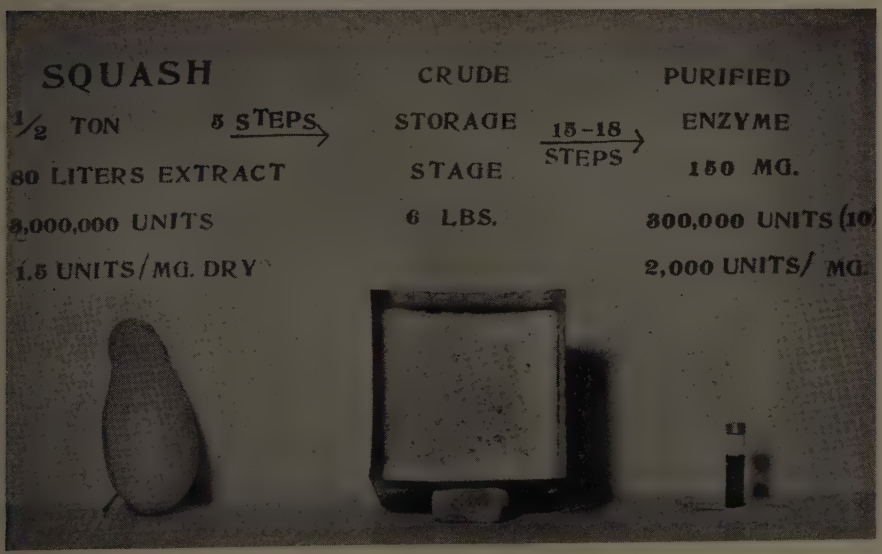


FIGURE 2. The purification of ascorbic acid oxidase from summer crookneck squash.

centrated solutions of the enzyme are stable for relatively long periods of time at physiological pH, both the activity and copper content are rapidly lost in parallel fashion when the enzyme is exposed to an acidity greater than about pH 4 (FIGURE 5). Second: the enzyme activity and copper content are not affected by certain strong copper chelating agents, notably ethylene diamine tetraacetate (EDTA) and amberlite resins, but are sensitive to agents such as cyanide ion, sulfide ion, and diethyldithiocarbonate. Dialysis against cyanide produces an inactive apoenzyme that can be partially reactivated by the

addition of copper.[11] Third: the copper of the enzyme is not exchangeable with ionic copper except when the enzyme is functioning as a catalyst.[12] When this observation was first made several years ago, it was not possible to decide whether the exchange was dependent on the enzyme's activity or was the result

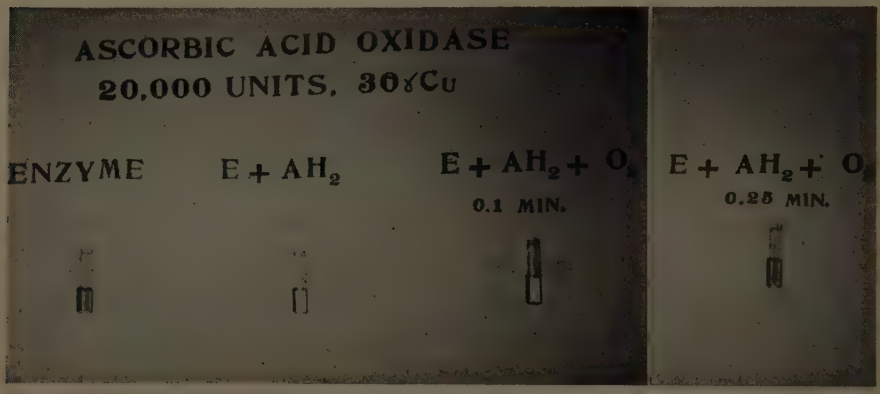


FIGURE 3. The enzyme is bleached by its substrate, ascorbic acid. Oxygen restores the blue color.

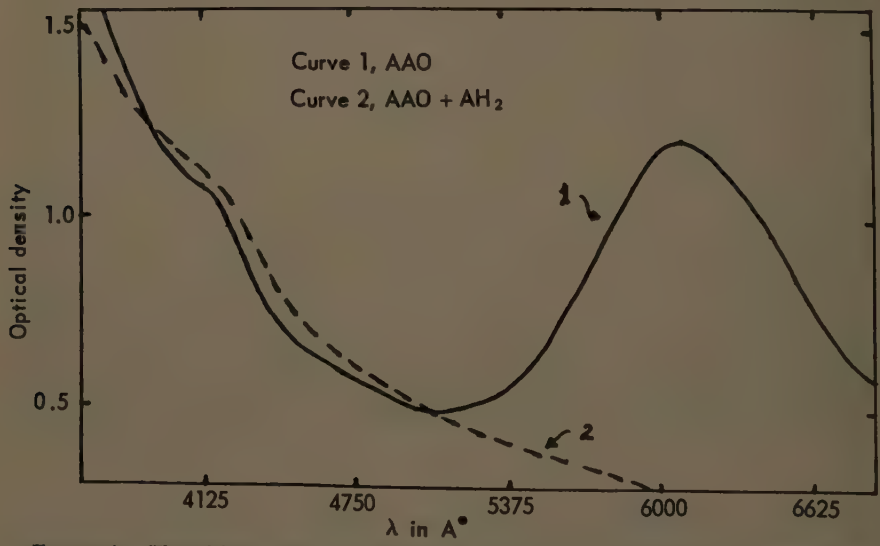


FIGURE 4. The visible absorption spectra of ascorbic acid oxidase in the presence and absence of substrate.

of enzyme inactivation which is always associated with the catalytic function. More recent studies in our laboratories now have made it clear that the inactivation process is not the causative factor (R. J. Magee and C.R. Dawson; to be published).

It may now be useful to return to the question of the possible chelate struc-

ture of this copper-protein complex. It is well known that ionic copper forms complexes with ammonia, amino acids, peptides, proteins, and other organic materials containing basic (electron-donating) nitrogen, and oxygen-containing

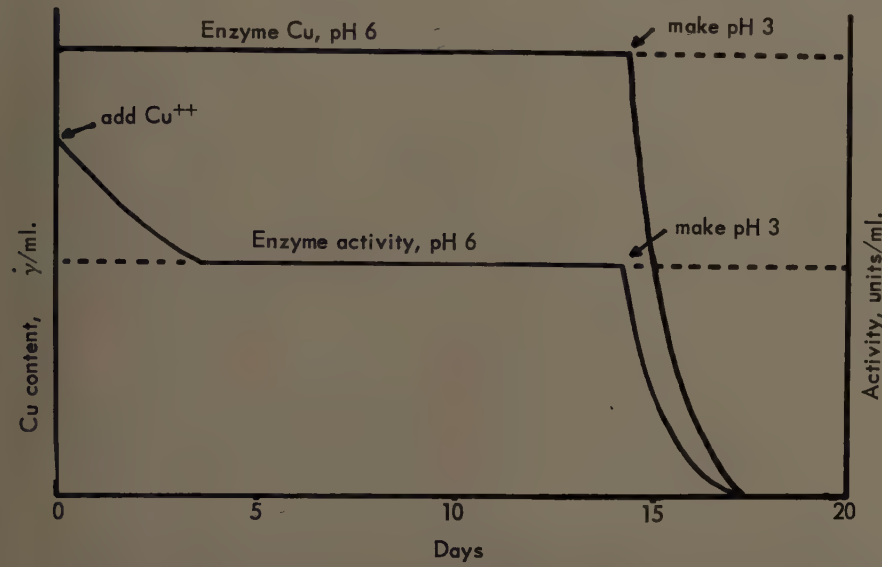


FIGURE 5. Showing the stability of the enzyme's copper content and activity to dialysis at physiological *p*H. Horizontal scale gives the days of dialysis. Note that both the activity and copper content are rapidly lost when the system is acidified to *p*H 3.

TABLE 2

THE PRINCIPAL VISIBLE ABSORPTION OF CUPRIC ION AND SELECTED NITROGEN COMPLEXES[13]

Complex	Molecular extinction	μ	*p*H
Cu^{++}	6	690	*
	12	800	†
$Cu(NH_3)_4^{++}$	55	606	—
$Cu(imidazole)_4^{++}$	53	590	7.2†
$Cu(glycine)_2^{++}$	19	780	3.1
	47	630	8.4
$Cu(triglycine)_2^{++}$	70	610	7.5
$Cu(tetraglycine)_2^{++}$	95	580	7.5
Cu^{++}-β-lactoglobulin	19	ca. 800	5.5
	58	580	9.8
Cu^{++}-bovine serum albumin	25	740	4.5
	52	680	7.0

* Aqueous cupric chloride, 0.01 *M*.
† Data from Edsall *et al.*[14]

groups. The visible absorption spectra of such copper complexes show a maximum absorption that may lie anywhere between about 600 and 800 mμ, dependent on the *p*H (TABLE 2). The principal absorption of 0.01 *M* cupric chloride solution occurs at 800 mμ. However, as ammonia or an amino acid (or peptide) is added, the position of maximum absorption shifts to shorter wave length, and

higher molecular extinctions are obtained. Thus the tetra-coordinated ammonia complex has its maximum absorption at 606 mμ with a molecular extinction of 55, which is comparable to the tetra-coordinated imidazole complex. Klotz and his co-workers[13] have concluded that the absorption peak of cupric ion in complexes shifts to shorter wave lengths as the coordination number is increased and exhibits maximum absorption in the 600 mμ region when the copper is tetra-coordinated. It is of interest to note also (TABLE 2) that the absorptions of the $Cu(glycine)_2^{++}$ complex and the complexes made by adding cupric ion to protein, that is, β-lactoglobulin and bovine serum albumin, shift to shorter wave length and higher extinction as the solution becomes more alkaline. In view of this *p*H effect, it is of particular interest to note that the naturally occurring copper proteins exhibit their principal absorption in the neighborhood of 600 mμ over a relatively wide *p*H range; 5.5 to 9.6 (TABLE 3).

On the basis of the spectrophotometric data just reviewed, it seems obvious

TABLE 3
THE PRINCIPAL VISIBLE ABSORPTION OF SOME COPPER-PROTEIN COMPLEXES FOUND IN NATURE

Complex	Molecular extinction	μ	*p*H	Ref. No.
Copper-serum protein (man)	1172*	605	5.5–8.0	15
(pig)	1215*	605	5.5–8.0	15
Oxyhemocyanin from				
Busycon canaliculatum (conch)		570	9.2–9.6	16
Limulus polyphemus (horseshoe crab)		590	6.1–9.4	16
Homarus americanus (lobster)		570	7.8–9.1	16
Loligo paelei (squid)		590	8.1	16
Ascorbic acid oxidase	767†	606	5.6	17

* Molecular extinction per copper calculated for a molecular weight of 151,000 and 8 copper atoms. Experimental values, 9380 (man), 9720 (pig).

† Molecular extinction per copper calculated for a molecular weight of 146,000 and 6 copper atoms. Experimental value, 4600.

that the type of bonding between the copper and protein of ascorbic acid oxidase and the other naturally occurring copper proteins, characterized by very high extinction at about 600 mμ over a relatively wide *p*H range, is very different from that causing the absorption of much lower extinction and *p*H-dependent wave length when Cu^{++} ion is merely added to nonspecific protein matter. It seems likely that 2 fundamental aspects of this difference involve an increased degree of coordination of the copper and an increased potentiality of resonance stabilization in the specific ligand systems of the natural copper protein complexes. Another factor that may contribute to the enhanced extinction of the copper oxidase has also been suggested,[17,18] that is, that the copper in the resting (nonfunctioning) enzyme may exist in 2 valence states: cupric and cuprous. McConnell and Davidson[19] have shown that complexes containing Cu^{I} and Cu^{II} have higher optical densities than either Cu^{I} or Cu^{II} complexes. Hemocyanin has copper initially in the Cu^{I} state and is colorless. When oxygenated, one half of the copper is oxidized to the Cu^{II} state, and the solution turns blue.[20] The question as to whether or not oxygen may play a similar role in the blue

color and activity of ascorbic acid oxidase cannot be answered at this time. However, experimental work is in progress.

All of the evidence thus far accumulated favors the view that the copper of ascorbic acid oxidase is probably bonded primarily to nitrogen atoms. All of the sulfur in the native enzyme can be accounted for in terms of methionine and cystine, and no free sulfhydryl groups can be detected (*see below*). These facts appear to rule out sulfur as a primary bonding group for copper. Furthermore, copper-sulfur bonds at *p*H 5.6 are too stable to be useful in explaining the properties of the enzyme. The stability characteristics of oxygen-copper bonds involving the oxygen atom of hydroxyl or carbonyl groups do not seem attractive, and copper-oxygen coordination complexes involving carboxylate groups can be ruled out, since copper-carboxylate interactions never show absorption below 720 mμ.

Of the several nitrogen-containing groups found in proteins that might be involved in the copper chelation, the following are of interest:

	*p*K
the α-amino group	7.6 to 8.4
the ε-amino group	9.4 to 10.6
the quanidinium group	11.6 to 12.6
the imidazolium group	5.6 to 7.0

At a *p*H of 5.6 all of these groups, except the imidazolium group, would exist, for the most part in the protonated form. In other words, only the imidazolium group would exist at *p*H 5.6 in a form suitable for copper binding. Thus it seems plausible to account for the absorption at 606 mμ for the blue solution of ascorbic acid oxidase at *p*H 5.6 in terms of a chelate complex between the copper and several (probably 2 to 4) imidazole groups of histidine residues suitably placed in the protein structure.

I now mention very briefly some results that have been obtained recently in our laboratory concerning the possible role of sulfhydryl groups in ascorbic acid oxidase activity.[21] Inhibition studies with *p*-chloromercuribenzoic acid have led to the conclusion that the activity of the pure enzyme is *not* sulfhydryl-dependent, an observation that contradicts the earlier reports of Frieden and his co-workers.[22-25]

Furthermore, no free —SH groups could be detected in the native enzyme using an ampermetric titration method which determined as little as 0.01 micromole of —SH and revealed 10.8 ± 0.4 sulfhydryl groups per mole of enzyme after denaturation. When the residual disulfide bonds of the denatured enzyme were reduced, the value for —SH per mole was found to be 17.9 ± 1.9 by ampermetric titration. This value is in very good agreement with the value 17.8 found for half-cystine residues per mole when the pure enzyme was hydrolyzed and when the amino acid composition of the hydrolysate was quantitatively determined by the chromatographic procedures described by Spackman and his co-workers.[26] The quantitative determination of the amino acid composition of ascorbic acid oxidase revealed 18 different amino acids and a significant amount (10.6 residues per mole) of the amino sugar glucosamine.[21]

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USE OF SYNTHETIC CHELATING AGENTS IN PLANT NUTRITION AND SOME OF THEIR EFFECTS ON CARBOXYLATING ENZYMES IN PLANTS*

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Introduction

Synthetic chelating agents in recent years have beome a reasonably satisfactory means of supplying micronutrients (iron, zinc, manganese) to plants. Sometimes chelating agents have effected growth responses beyond those that could be attributed to micronutrients. The postulated explanations include auxinlike effects and also improved micronutrient balance; another is an *in vivo* stimulation of some of the reactions relating to photosynthesis by chelating agents accumulated by plants. The carboxydismutase enzyme is extremely sensitive to heavy metals. Chelating agents protect against heavy-metal inactivation of a large number of enzymes in *in vitro* studies. In cell-free preparations many chelating agents not only increase the amount of CO_2 fixation, but also overcome the inhibition caused by the added heavy metals. Weissbach *et al.*[1] were first to report that chelating agents increased CO_2 fixation with the carboxydismutase enzyme.

A brief review of some of the behavior of synthetic chelating agents and their metal chelates in plants will clarify the studies in this laboratory on carboxylating enzymes.

Synthetic Chelating Agents in Plant Nutrition

Synthetic chelating agents were used in the nutrition of microorganisms[2] before their use was developed for higher plants. EDTA and other chelating agents have given excellent results in maintaining adequate levels of micronutrient elements in nutrient substrates for plants over relatively long periods of time.[3] In addition, high or even moderate levels of micronutrient elements are less toxic to plants if a chelating agent is present in the nutrient solution.[4] The usual result is that media containing chelating agents give higher yields than do those without chelating agents, although they have equal amounts of micronutrients. A most interesting effect of EDTA on algal growth was noted by Walker.[5] EDTA relative to no EDTA decreased yields when levels approaching a deficiency of zinc and manganese were supplied; it had no depressing effect, however, when levels approaching a deficiency of iron were used. Either the zinc and manganese EDTA were absorbed less readily by the algae

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The abbreviations used in this report are: EDTA, ethylenediaminetetraacetic acid; EDDHA, ethylenediaminedi-*o*-hydroxyphenylacetic acid; HEEDTA, hydroxyethylethylenediaminetriacetic acid; R5P, ribose-5-phosphate; TPN, triphosphopyridine nucleotide; PEP, phosphoenolpyruvate; RDP, ribulose diphosphate; PGA, 3-phosphoglyceric acid; GSH, reduced glutathione; *p*-CMB, parachloromercuribenzoate; ATP, adenosine triphosphate; OAA, oxaloacetic acid; μmole, micromole (10^{-6} mole).

than was the iron EDTA or the iron was separated more readily from the EDTA inside the cells than were zinc and manganese. The possible relationship of this to higher plants will be seen below.

Following the use of EDTA in nutrient solutions to maintain iron in a soluble form,[6] the iron chelates came into widespread use for correcting iron deficiency in plants by their application to soil[7] (there is a literature of several hundred references on the subject). Foliage application is also made, but less extensively and somewhat less successfully than soil application. Economic considerations, however, dictate low application rates and limit the use of chelates.

TABLE 1*

DRY WEIGHT YIELD AND FE CONTENT OF SOYBEAN SEEDLINGS AS INFLUENCED BY EDDHA IN SAND CULTURE†

Iron level and source (ppm)	Dry weight yield/plant (gm.)	Fe content of dry weight (ppm)
None	1.26	133
5 $FeSO_4$	1.36	223
50 $FeSO_4$	1.21	389
5 FeEDDHA	1.64	169
50 FeEDDHA	1.76	370
Equivalent 5 Fe as EDDHA	1.56	174
Equivalent 50 Fe as EDDHA	1.63	127

* Part of TABLE 6, Wallace *et al.*[4]
† None of the plants had an iron deficiency.

TABLE 2*

YIELDS OF BEAN PLANTS GROWN IN A CALCAREOUS SOIL WITH IRON CHELATES†

Fe chelate‡	Fresh weight of plants (gm.)
None	18.7
EDTA	12.3
DTPA§	15.8
HEEDTA	11.0
EDDHA	24.5

* Part of TABLE 4, Wallace *et al.*[17]
† None of the plants was iron-chlorotic.
‡ The iron supplied was 175 mg. for a 500-gm. quantity of soil.
§ Diethylenetriaminepentaacetate.

In some cases zinc and manganese deficiencies in higher plants have been corrected successfully with appropriate chelates.[3] Instability in soil is a problem in the use of zinc and manganese chelates.

Examples of improved plant yields from the use of metal chelates under conditions in which no micronutrient deficiencies exist are given in TABLES 1 and 2. The fact that chelating agents behave like auxins[8] has been shown to be doubtful as an explanation.[9] That the depressing effect of some iron chelating agents on absorption of such micronutrients as manganese sometimes results in an improved balance of micronutrients[10] is still a possible explanation. This phenomenon will be described in detail. The principal reasons for suspecting

that chelating agents have a net beneficial effect on enzyme reactions in plants, as another possible explanation of the yield increase, is the evidence that some reactions have increased activity in the presence of chelating agents and that the chelating agents are absorbed by plant roots and translocated to leaves when used in plant nutrition.

Wallace and North[11] first showed that both metal and chelate are absorbed by plant roots and translocated to the foliage, although Wallace *et al.*[4] later indicated that iron and chelating agents were present in leaves in different amounts after root application. Most of the data from this laboratory indicate that more chelating agent than metal, when both were tagged with isotopes,

TABLE 3
EDTA AND IRON RATIOS IN PLANT PARTS FOLLOWING ROOT APPLICATION OF C^{14}- AND Fe^{59}-LABELED MATERIAL

Plant	EDTA/Fe ratios*		
	Leaf	Stem	Root
Soybean	3.6	2.5	0.9
Pyracantha	6.8	5.0	0.4
Rough lemon	4.1	3.5	1.1

* Calculated from specific activities. Single salt solutions containing 5 ppm iron were supplied for 5 days in sand culture.

TABLE 4
EDTA AND ZINC RATIOS IN PLANT PARTS FOLLOWING ROOT APPLICATION OF C^{14}- AND Zn^{65}-LABELED MATERIAL

Plant	EDTA/Zn ratios*		
	Leaf	Stem	Root
Soybean	4.2	2.2	1.2
Pyracantha	6.0	2.5	1.3
Rough lemon	5.0	3.0	0.2

* Calculated from specific activities. Single salt solutions with 5 ppm zinc were supplied for 5 days in sand culture.

reached the leaves after soil application (TABLES 3 and 4). Recent unpublished studies with Fe^{59}- and C^{14}-labeled EDDHA, which forms an extremely stable iron chelate, have indicated sometimes equimolar amounts of the chelate and iron in leaves. Tiffin and Brown,[12] however, reported recently that the chelating agent remained in the nutrient solution and that only the metal was absorbed. In cases in which a free chelating agent without a chelated metal has been transported to leaves (sodium salts of chelating agents supplied to roots appear to translocate readily to leaves) or in which such are separated from metal chelates in roots or in leaves after translocation, it is expected that they will have important effects on plant metabolism through chelation of metals present in leaves, if the chelating agents themselves are not metabolized.

There is little evidence that synthetic chelating agents are metabolized in

plants. Although iron chelating agents undergo oxidation in the presence of sunlight,[13] and leaves are exposed to sunlight, there is no direct evidence that this oxidation is the manner in which chelating agents decompose in plants.

Metal chelates appear to be absorbed slowly or not at all by the roots of some plant species.[14] These plants, of course, do not respond to the applications. What is involved in the absorption failure is not known.

Moderate to high levels of heavy metals are toxic to plants. The addition of certain chelating agents to the media containing such levels of heavy metals often overcomes some or all of the toxicity. This is illustrated in TABLE 5. Heavy metals may be detoxicated either by preventing their absorption by

TABLE 5*

EFFECT OF EDDHA WITH DIFFERENT MICRONUTRIENT LEVELS ON SOYBEAN YIELDS

Nutrient variables	Without EDDHA (gm. dry weight)	With 0.0015 *M* EDDHA (gm. dry weight)
No Fe	1.39	1.77
No Fe + HCO_3^-	1.77	2.22
High Mn and Zn	1.00	2.16
High Cu and P	0.76	1.50
L.S.D. (0.05)†	0.57	

* Part of TABLE 5, Wallace *et al.*[4]

† Least significant difference at the 5 per cent possibility of error.

TABLE 6

FeEDDHA EFFECT ON YIELD AND MICRONUTRIENT CONTENT OF SOYBEANS GROWN IN A CALCAREOUS SOIL

Lbs./acre equivalent Fe as EDDHA	Yield (gm.)	Fe (ppm)	Mn (ppm)	Zn (ppm)
0	1.39	40	88	77
5	1.64	41	11	86
200	1.54	359	7	97
L.S.D. (0.05)*	0.21	20	13	18

* Least significant difference at the 5 per cent possibility of error.

plants or by chelation after they are in plants. The relative importance of each is not known.

Iron chelates applied to plants often induce manganese deficiencies.[15] Iron chelates actually hinder the absorption of manganese by plants (TABLE 6). This can be a practical means of overcoming toxicity such as that caused by manganese, as was successfully done in coffee.[16] The nature of this inhibition of absorption of manganese thus far has resisted investigation.

Some chelating agents are toxic to plants, and often the line between adequacy to correct a nutrient deficiency and that producing toxicity is narrow. EDDHA has been the least toxic of the synthetic chelating agents,[17] plants being able to grow well in the presence of large quantities of it. Under iron deficiency conditions, however, a point is reached at which this chelating agent

is quite lethal (TABLE 7). This indicates the metabolic effects of the chelating agent within a plant.

Iron chelating agents have masked the symptoms of some virus diesases in plants.[18] In camellia, symptoms of chlorophyll deficiency disappeared from the leaves, and virus symptoms also disappeared from flowers that were red and white under the virus condition but a solid red when large applications of iron chelates were made. Whether such effects are due solely to iron or in part to the chelating agent is not known.

Review of Literature on Effect of Chelating Agents on Carboxylating Enzymes in Plants

Weissbach *et al.*[1] showed that a chelating agent or an agent with one of the —SH groups, such as GSH, was necessary for maximal activity of the carboxydismutase enzyme. They implied that both agents used in inactivating heavy metals served to protect the —SH groups on the enzyme.

Huffaker *et al.*[19] found that chelating agents enhanced activity for the CO_2 fixation catalyzed by the PEP carboxylase enzyme. They strengthened the

TABLE 7*

YIELDS OF BUSH BEAN SEEDLINGS GROWN AT IRON LEVELS AND WITH 0.0015 *M* CHELATING AGENTS

Fe in nutrient solution (ppm)	No chelate (gm.)	EDDHA (gm.)	HEEDTA (gm.)
0	4.8†	3.0	5.7
5	4.9	13.3	2.8

* Part of TABLE 5, Wallace *et al.*[4]

† Very chlorotic plants.

case for the protection against heavy metals by showing that preparations from plants pregrown with slight iron deficiencies resulted in higher amounts of CO_2 fixation in cell-free preparations with either a PEP or an R5P (R5P → RDP) reaction system than did those from plants pregrown with adequate iron. A chelating agent added to reaction systems further increased CO_2 fixation. The chelating agent also overcame the inhibition for both systems that was caused by the *in vitro* addition of iron to the preparations.

In further studies, Huffaker and Wallace[20] showed that, for the same two reaction systems, inhibition was caused by the *in vitro* addition of molybdenum, copper, zinc, and manganese. The addition of EDDHA to the preparations overcame part of the inhibition caused by zinc, copper, and manganese. Pregrowing plants with high levels of micronutrients resulted in decreased activities, which could be increased somewhat by the *in vitro* addition of EDDHA to the reaction systems. Plants pregrown with EDDHA as well as with high levels of micronutrients resulted in higher amounts of CO_2 fixation, particularly that catalyzed by the carboxydismutase system, than similar plants not pregrown with EDDHA. This effect may be of great importance and may relate to the phenomenon, mentioned above, of unmetabolized chelating agents accumulating in parts of plants.

An interesting report that may relate to CO_2 fixation in photosynthesis concerns a very large stimulation by EDTA of the glyceraldehyde-3-phosphate dehydrogenase-TPN-requiring enzyme, which catalyzes the reduction of PGA to the aldehyde.[21] This is the carbon reduction step in photosynthesis, the source of the reduced TPN being photoreduction of oxidized TPN. This PGA reduction follows the formation of 2 molecules of PGA from the enzymatic combination of CO_2 with RDP. A possible relationship of this EDTA effect to equilibrium conditions in this reaction sequence may have important implications in growth effects.

As mentioned above, certain chelating agents have proved toxic to plants. The chelating agent HEEDTA showed more toxicity than any other (TABLE 7) and also was found to inhibit CO_2 fixation with both PEP and R5P as substrates more than any other.[22] Lineweaver-Burke double-reciprocal plots with varying R5P as a substrate indicated a noncompetitive type of inhibition. Additional studies of this effect are reported below.

Purpose of Study

Since some chelating agents, especially EDDHA, often result in yield increases beyond the effect of micronutrient supply, since the chelating agent itself can accumulate in plants, and since chelating agents have been shown to have pronounced effects on activities of several enzyme reactions including those of carboxylating enzymes, more information is needed concerning the nature of the effects of synthetic chelating agents on reactions catalyzed by specific enzymes.

Experimental Methods

The reagents were prepared as follows:

A stock solution of 0.01 *M* of the cyclohexylamine salt of PEP was prepared in 0.2 *M* Tris [tris(hydroxymethyl)aminomethane] buffer, *p*H 8.0. The K salt of R5P was prepared from the Ba salt by precipitating the Ba with an equivalent amount of K_2SO_4. The reagent was centrifuged to remove the precipitate, and stock solutions of 0.02 *M* were prepared in Tris buffer as above. Stock solutions of 0.01 *M* Na salts of chelating agents were prepared and made to *p*H 8.0.

Bush bean seeds and corn were germinated in sand and allowed to grow for about 10 days to about 2 inches in length. Recently matured sweet orange leaves, used in some studies, were obtained from trees in the orchard at the University of California.

The assays were made as follows:

Leaf homogenates were produced by grinding 1 weight of fresh leaves with 3 vol. of Tris buffer, 0.2 *M* at *p*H 8.0, with a mortar and pestle at 0° C. The homogenate was strained through 2 layers of cheesecloth and kept at 0° C. until used, this storage period never exceeding one-half hour. All the reaction mixtures received 140 μmoles $KHC^{14}O_3$ containing 1.2×10^6 counts per minute (cpm) as the $BaCO_3$ precipitate with a Q-gas counter, and 0.1 ml. enzyme preparation, which was added last. The total volume of each reaction mixture was 1.0 ml. before addition of acid. Except where noted oth-

erwise, the reaction mixtures contained the following, where appropriate, for the 1.0 ml. of reaction mixture (in micromoles); 1 PEP, 2 R5P, 20 Mg, and 4 ATP. The mixtures were incubated for 10 min. at 37° C., and the enzyme was killed with 0.1 ml. 1 *N* HCl, which contained 2,4-dinitrophenylhydrazine to convert any OAA present to the hydrazone form. Such a system was found to prevent almost completely the decarboxylation of labeled products during the period when the planchets were dried prior to counting. This also expelled unreacted HCO_3^-. The mixtures were centrifuged, and aliquots of 0.2 ml. of the supernatant then were dried in forced air at room temperature in Pyrex planchets and were counted. The results were reported in counts per minute in this aliquot. This method was described by Jackson and Coleman[23] as a modification of that employed by Bandurski and Greiner[24] and by Saltman *et al.*[25]

The coefficient of variability of this method was found to be less than 4 per cent, and this value is taken into account in the discussion of the results. All studies were repeated several times, and data are included that represented consistent trends.

Two carboxylating systems were studied. The first system catalyzed by PEP carboxylase was represented by the following reaction systems:

$$PEP + HCO_3^- \xrightarrow{Mg} OAA + H_2PO_4^-$$

The second system catalyzed by carboxydismutase (carboxylation enzyme) was represented by the following reaction system:

$$R5P + ATP + HCO_3^- \xrightarrow{Mg} 2\ PGA + ADP$$

Actually, the latter is a 3-enzyme step that requires also the phosphoriboisomerase and phosphoribulokinase enzymes.

Although the general trends observed were consistent, there was considerable day-to-day variation in the magnitude of the responses, which is evidently in part the result of the method.

EDDHA was used in most of the studies because it was the most successful iron chelate in plant nutrition, although it was not the agent that effected the most positive response with the PEP system.[22] Unless otherwise noted, the quantity of EDDHA used in 1.0 ml. of reaction mixture was 1 μmole.

Effect of EDDHA on Heavy-Metal Inhibition of Carboxylating Enzymes

Iron and other heavy metals supposedly are inhibitors of the 2 carboxylating reactions.[19,26] It is not known whether, when the chelating agent is added, the increased C^{14} in fixation products—always observed when R5P was the substrate for CO_2 fixation and often observed when PEP is the substrate—is the result of chelation of endogenous heavy metals. When certain heavy metals were added, however, EDDHA did restore some and in some cases all of the decrease in C^{14} in fixation products for both R5P (TABLE 8) and PEP (TABLE 9) systems. GSH had an effect similar to EDDHA, except that it had more effect than EDDHA on overcoming Cu and Zn inhibition of the R5P system. GSH did not overcome Cu inhibition of the PEP system.

A series of studies was made to determine the following: whether the effect

of the EDDHA was to prevent metal inhibition of the enzyme reaction or whether the chelate merely prevented decarboxylation of C^{14} acids after they had been synthesized and, particularly, during the drying of them on planchets; and why decarboxylation of fixed products should occur when the hydrazone of OAA was formed in the PEP reactions to prevent such losses.

In TABLE 10 the data for the R5P reaction, and under the experimental conditions, with sweet orange leaf preparations, indicate that EDDHA, when present in the reaction mixture, essentially prevented zinc inhibition. In contrast, the addition of the EDDHA after the reaction but before drying had a slight effect when only zinc but no chelate had been added to the reaction mixture.

TABLE 8

EFFECT OF EDDHA IN OVERCOMING INHIBITION OF SOME HEAVY METAL SALTS ON $C^{14}O_2$ FIXATION WHEN R5P WAS USED AS A SUBSTRATE IN CELL-FREE PREPARATIONS OF BUSH BEAN LEAVES*

Heavy metal salts added (1 μmole)	EDDHA (cpm/aliquot)		
	None	1 μmole	2 μmoles
None	4680	5760	3940
Cu	62	154	2920
Mo	2790	3400	2900
Zn	35	90	2360

* *See* under *Experimental Methods* for procedure.

TABLE 9

EFFECT OF EDDHA AND GSH ON HEAVY METAL INHIBITION OF THE PEP CARBOXYLASE REACTION IN PREPARATIONS FROM SWEET ORANGE LEAVES*

Heavy metal salts	(μmole)	Nothing used (cpm/aliquot)	EDDHA (cpm/aliquot)		GSH (cpm/aliquot)
			1 μmole	3 μmoles	1 μmole
None	—	2500	2550	2850	2310
Cu	1.0	1210	2230	3140	600
Hg	0.1	370	410	1420	2660
Mn	1.0	2310	2450	3310	2450

* *See* under *Experimental Methods* for procedure.

EDDHA added after the reaction and before the drying did increase considerably the C^{14} count in each case. This indicates the presence of unstable products that decompose in the presence of nonchelated zinc and other metals. In part, the effect of the chelating agent is to prevent such decomposition.

The data for PEP with sweet orange (TABLE 10) indicate that EDDHA, at least in part, does not necessarily overcome a zinc inhibition of the enzyme, but rather that it overcomes a zinc-induced decarboxylation of fixed product. With corn, however, the data indicated an effect of zinc on the enzyme reaction.

Our studies never have indicated an EDDHA stimulation of the PEP reaction with preparations from roots. Data in TABLE 11 indicate an EDDHA inhibition of the reaction with preparations from bush bean roots and also a

constant degree of inhibition by the chelating agent obtained with and without manganese. Manganese also inhibited the reaction. This result is different from that obtained from the preparations from leaves.

TABLE 10

EFFECT OF ADDING EDDHA TO REACTION MIXTURES AFTER THE REACTION PERIOD AND JUST BEFORE ADDITION OF THE ACID TO STOP CO_2-FIXING REACTIONS WITH R5P AND PEP SYSTEMS WITH PREPARATIONS FROM LEAVES*

Additions during reaction	Amounts of additions (μmole)	Regular procedure (cpm/aliquot)	EDDHA just before acid (cpm/aliquot)
			1 μmole
Sweet orange leaves			
R5P reactions			
—	—	7050	7940
EDDHA	1	9080	9790
$ZnSO_4$	0.2	255	446
EDDHA + $ZnSO_4$	1 + 0.2	7970	9000
PEP reactions			
—	—	2340	2780
EDDHA	1	2360	—
$ZnSO_4$	0.2	1970	2270
EDDHA + $ZnSO_4$	1 + 0.2	2450	—
			10 μmoles
Corn leaves			
PEP reactions			
—	—	1100	2500
EDDHA	1	998	1195
$ZnSO_4$	0.5	404	520
EDDHA + $ZnSO_4$	1 + 0.5	874	910

* *See* under *Experimental Methods* for procedure.

TABLE 11

MANGANESE AND EDDHA INHIBITION OF $C^{14}O_2$ FIXATION WITH PEP AS A SUBSTRATE IN PREPARATIONS FROM BUSH BEAN ROOTS*

Mn (μmole)	EDDHA (cpm/aliquot)		
	None	0.5 μmole	1 μmole
0	3940	2720	2610
1	2550	1940	1800
2	2300	1910	1620
5	1620	1200	1050

* No Mg was used. *See* under *Experimental Methods* for procedure with Mg.

The Nature of PEP Carboxylase and Carboxydismutase

Carboxydismutase has been shown to be a sulfhydryl enzyme,[1] as have been the isomerase and kinase enzymes that also are involved when R5P is used as a substrate.[21] PEP carboxylase is also suspected of being a sulfhydryl enzyme; metal inhibitors (TABLE 12) do not contradict this. The cyanide inhibition of the R5P reaction may be attributed to the formation of the cyanohydrin[27] with

the ribulose diphosphate rather than to metal inhibition, since azide failed to inhibit the reaction. With azide for both reactions there appeared to be stimulation. Although several possibilities exist, the stimulation could occur, at least for cyanide with PEP, through a combination with reaction products and thus prevent decarboxylation or, alternatively, it could be caused by cyanide combination with heavy metals, thus rendering them nontoxic.

Studies were made to determine whether EDDHA would be helpful in characterizing the 2 reaction systems. Under the study conditions used, GSH was able to overcome the inhibition resulting from 10^{-4} *M* *p*-CMB for both the PEP and R5P reactions, but EDDHA was not able to do so (TABLE 13); *p*-CMB

TABLE 12

EFFECT OF INHIBITORS ON CARBOXYLATING REACTIONS IN PREPARATIONS FROM SWEET ORANGE LEAVES

Inhibitor*	PEP (cpm/aliquot)	R5P (cpm/aliquot)
Control	3350	4210
Azide	3500	5270
Fluoride	3660	3880
Cyanide	5890	274

* Inhibitor concentration was 10 μmoles. *See* under *Experimental Methods* for procedure.

TABLE 13

ABILITY OF GSH BUT NOT EDDHA TO OVERCOME INHIBITION OF *p*-CMB ON CARBOXYLATION REACTIONS

p-CMB (μmole)	Control (cpm/aliquot)	EDDHA, 1 μmole (cpm/aliquot)	GSH, 1 μmole (cpm/aliquot)
Bush bean roots, PEP			
0	3820	3820	3820
0.1	1400	1190	3820
Bush bean leaves, PEP			
0	3310	3310	3310
0.1	900	874	3220
Sweet orange leaves, R5P			
0	910	7470	3600
0.1	41	22	3400

is an alkylating agent. These results indicate a similar nature of the enzymes involved.

That the 2 systems may be fundamentally different, however, is suggested by some of the data in TABLES 14 and 15. For example, EDDHA and GHS greatly stimulated the R5P system, but usually had less or no effect on the PEP system. Phosphate at 3×10^{-2} *M* stimulated the PEP reaction, but not the R5P. Ammonium sulfate at 5×10^{-2} *M* inhibited the R5P reaction, but not the PEP. The omission of Mg and the addition of *p*-CMB, $HgCl_2$, and iodoacetate inhibited the R5P more than the PEP reaction. The heavy metal inhibition studies, some of which have been referred to, usually showed considerably greater inhibition of R5P than of PEP. Part of this effect, however, could be due to the fact that the R5P system is a 3-enzyme system.

$Ca(NO_3)_2$ resulted in greater inhibition of the R5P reaction than the PEP, but this is possibly[28] the effect of Ca on ATP.

Effect of EDDHA on Salt Inhibition of the R5P Reaction

Salts at relatively high concentrations inhibit the R5P reaction system.[29] Calcium nitrate is particularly inhibiting, partly as a result of Ca reactions with

TABLE 14

EFFECT OF EDDHA IN THE PRESENCE OF DIFFERENT CHEMICALS ON THE PEP CARBOXYLASE REACTION*

Additions	Amount (μmole)	EDDHA (cpm/aliquot)					
		Bush bean roots			Sweet orange leaves		
		None	2 μmoles	5 μmoles	None	2 μmoles	5 μmoles
Control	—	1690	1430	854	3940	5060	4210
KH_2PO_4	10	1820	1410	841	4680	4210	3710
KH_2PO_4	30	2230	1670	990	3940	3310	2980
$NaHAsO_4$	20	2010	1740	1020	3400	4070	3390
$HgCl_2$	1	94	65	39	24	26	26
p-CMB	1	331	147	81	20	33	24
$Ca(NO_3)_2$	50	349	312	210	1220	2500	2350
$(NH_4)_2SO_4$	100	1340	1130	835	1670	2230	1800
Iodoacetate	10	1340	1060	735	1970	4200	3300

* *See* under *Experimental Methods* for procedure.

TABLE 15

COMPARISONS OF PEP AND R5P SYSTEMS IN CELL-FREE PREPARATIONS

Additions	Amount (μmole)	PEP + Mg + HCO_3^- (cpm/aliquot)	R5P + ATP + Mg + HCO_3^- (cpm/aliquot)	PEP + Mg + HCO_3^- (% of base reaction)	R5P + ATP + Mg + HCO_3^- (% of base reaction)
Soybean leaves					
Control	—	2720	319	100	100
EDDHA	1	2560	508	94	159
EDDHA	2	2660	525	98	164
GSH	1	2850	2140	105	670
KH_2PO_4	30	3500	339	128	106
$(NH_4)_2SO_4$	50	3060	108	112	34
$Ca(NO_3)_2$	25	453	93	17	29
Omit Mg	—	673	12	25	4
p-CMB	0.1	1400	20	51	6
$HgCl_2$	0.1	543	12	20	4
Iodoacetate	10	1800	111	66	35
Corn leaves					
Control	—	2150	7940	100	100
EDDHA	1	2560	10640	122	133
EDDHA	5	6040	14140	280	178
Sweet orange leaves					
Control	—	2360	5270	100	100
EDDHA	1	2450	6340	103	119
EDDHA	5	3400	10450	143	198

* *See* under *Experimental Methods* for procedure.

ATP, as mentioned.[28] Although EDDHA more than proportionally overcame the inhibition (TABLE 16), a Lineweaver-Burke double-reciprocal plot of the $Ca(NO_3)_2$ inhibition with and without the chelating agent (FIGURE 1) indicated a noncompetitive type of inhibition.

TABLE 16

$C^{14}O_2$ FIXATION IN CELL-FREE PREPARATIONS OF SWEET ORANGE LEAVES USING AN R5P + ATP + HCO_3^- REACTION SYSTEM WITH DIFFERENT LEVELS OF EDDHA AND WITH AND WITHOUT $Ca(NO_3)_2$ AS AN INHIBITOR*

Chelate level (μmole)	$Ca(NO_3)_2$			
	None (cpm/aliquot)	25 μmoles (cpm/aliquot)	None (%)	25 μmoles (%)
0	6870	1520	100	100
2	9130	3180	133	209
5	8610	3410	125	224

* *See* under *Experimental Methods* for procedure.

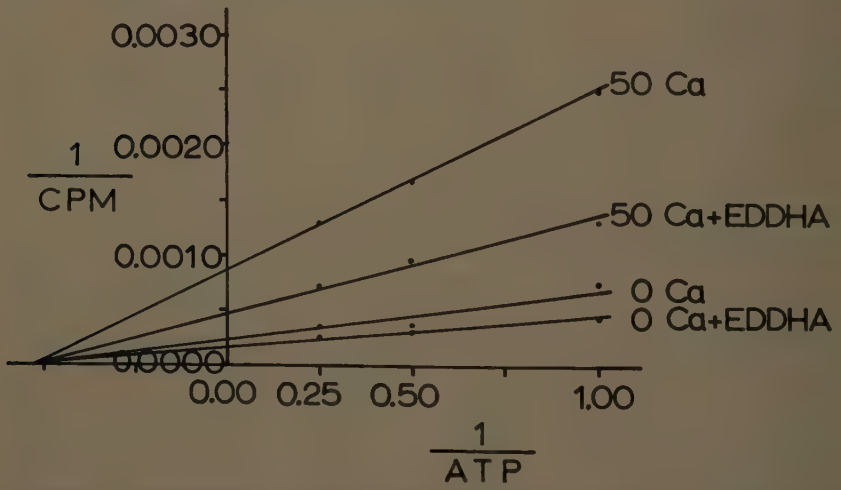

FIGURE 1. Double-reciprocal plot of $Ca(NO_3)_2$ inhibition and reversal of inhibition with EDDHA of $C^{14}O_2$ fixation with the R5P system with preparations from sweet orange leaves at varying levels of ATP. The concentration of EDDHA was 1 μmole for the 1.0 ml. of reaction mixture. Except as noted in the figure, the concentration of reactants was similar to that described under *Experimental Methods*.

Effects of HEEDTA on Carboxylation Enzyme Inhibition

A study was made (TABLE 17) to determine whether metals would overcome any of the toxicity of the chelating agent in the R5P reaction system. To a slight extent only, and usually on the R5P more than on the PEP reaction system, was there effect. This indicates that both the metal chelate and the chelating agent inhibit the system.

In addition to the reports of noncompetitive inhibition already made for

HEEDTA with the R5P reaction, determined with R5P as a substrate,[22] similar double-reciprocal plots were made for HCO_3^- and ATP as substrates. In each of these cases the noncompetitive inhibition also was indicated (FIGURES 2 and 3).

TABLE 17
EFFECT OF METALS ON TOXICITY OF CHELATING AGENT HEEDTA ON TWO DIFFERENT CO_2 FIXATION REACTIONS*

Salt*	Substrate (cpm/aliquot)	
	R5P	PEP
No chelate		
None	7470	2360
HEEDTA, 1 μmole		
None	771	442
$FeSO_4$	940	364
$ZnSO_4$	1220	693
$CuSO_4$	900	514
$CaCl_2$	1250	603

* One μmole of heavy metal salt and 50 μmoles of $Ca(NO_3)_2$ were added to the reaction mixtures where applicable. *See* under *Experimental Methods* for procedure.

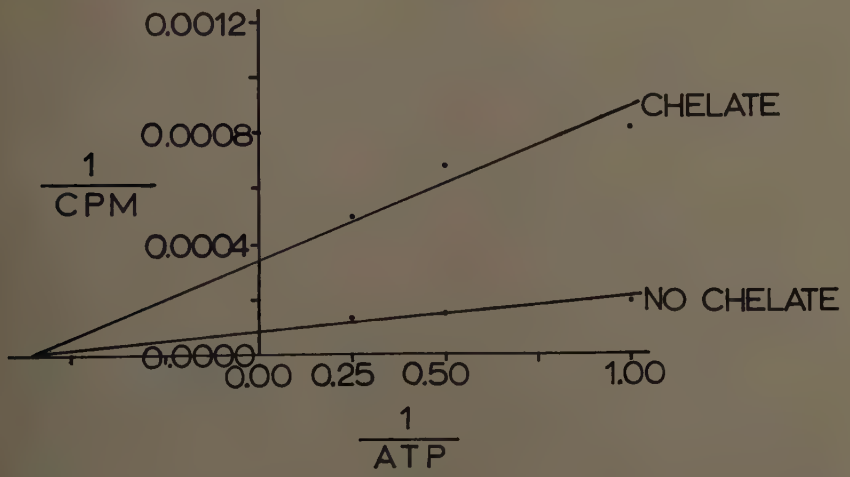

FIGURE 2. Double-reciprocal plot of HEEDTA inhibition of the R5P system in preparations from sweet orange leaves at varying levels of ATP. The concentration of chelating agent was one third μmole for the 1.0 ml. of reaction mixture. That of other reagents and enzymes was as indicated under *Experimental Methods*.

Effect of EDDHA on PEP Reaction in Etiolated Plants

Although EDDHA rather inconsistently increased C^{14} fixation with the PEP carboxylase system in preparations from green leaves, there was a consistent increase in activity with etiolated plants, as illustrated by the data in TABLE 18. The increase, however, was not great.

Effect of EDDHA on CO_2 Fixation at Different Magnesium Levels

Both reactions studied require Mg as a metal activator. Sometimes endogenous Mg is sufficient for maximal activity, especially in the PEP system. In the R5P system the relatively high level of ATP used may inactivate Mg. It was thought that the chelating agent might have inhibitory effects on the reactions under conditions of limiting Mg or that it might even render small levels of Mg more reactive. Data for the PEP reaction in a root preparation (TABLE 19) indicate that endogenous Mg was sufficient for maximal activity

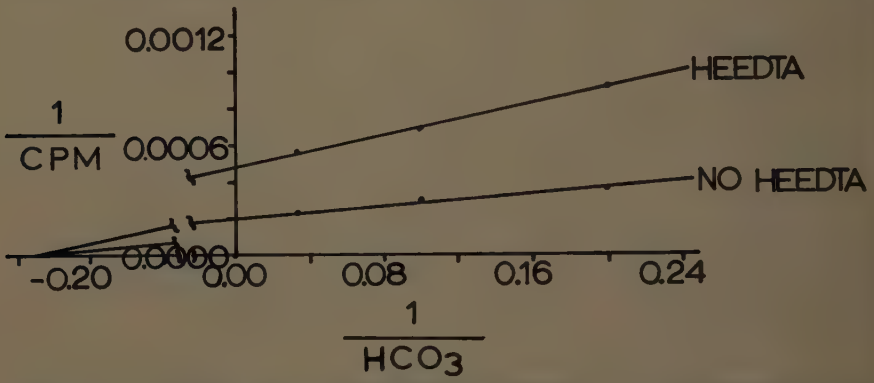

FIGURE 3. Double-reciprocal plot of HEEDTA inhibition of the R5P system in preparations from sweet orange leaves at varying levels of bicarbonate. The concentration of chelating agent was one third μmole for the 1.0 ml. of reaction mixture. That of other reagents and enzymes was as indicated under *Experimental Methods*.

TABLE 18
EFFECT OF ETIOLATION ON PEP REACTION SYSTEMS*

Reaction systems	Green (cpm/aliquot)		Etiolated (cpm/aliquot)	
	Bush bean	Corn	Bush bean	Corn
Minus substrates	387	135	82	194
PEP + Mg	4860	5760	6040	6050
PEP + Mg + EDDHA	4880	5720	6340	6400

* Seeds were germinated and left in a dark closet for 1 week. *See* under *Experimental Methods* for procedure.

when EDDHA was omitted, but not when it was present. Chelated Mg, then, was a hindrance to the reaction.

Similar data for the R5P system with leaf preparations (TABLE 20) indicate that there was a slightly less proportional increase for the EDDHA with endogenous Mg than when Mg was added. For all levels of added Mg, however, the same ratio of reaction with EDDHA and without EDDHA was obtained. This again indicates that chelated Mg hinders the reaction.

Summary

Heavy-metal inhibition of two carboxylation reactions studied in cell-free preparations of plant material was overcome, sometimes completely, by the

chelating agent EDDHA. Part of the effect, in the case of the system requiring R5P, was the result of preventing product decomposition during the drying of samples on the planchets. Most of the effect in the PEP system could be in preventing product decomposition. EDDHA and GSH consistently resulted in relatively large increases in $C^{14}O_2$ fixation in the R5P system, but in a less consistent influence on the PEP system. When heavy metals were added to the PEP system, the chelating agent usually was beneficial. Arsenate and phosphate stimulated the PEP, but not the R5P, reaction. Heavy metals, *p*-CMB, ammonium sulfate, $HgCl_2$, and the omission of Mg inhibited the

TABLE 19

EFFECT OF EDDHA ON THE PEP CARBOXYLASE REACTION IN BUSH BEAN ROOT PREPARATIONS AT DIFFERENT LEVELS OF MAGNESIUM*

Mg added (μmole)	EDDHA (cpm/aliquot)			Inhibition of EDDHA (%)	
	None	0.5 μmole	1 μmole	0.5 μmole	1 μmole
0 (endogenous only)	3940	2720	2600	31	34
2	4070	3500	3060	14	25
5	4680	3940	3220	16	31
20	4000	3710	3600	7	10

* *See* under *Experimental Methods* for general procedure.

TABLE 20

EFFECT OF MG ON CHELATING AGENT EFFECT ON CO_2 FIXATION WITH THE R5P + ATP + HCO_3^- REACTION SYSTEM WITH HOMOGENATE FROM BUSH BEAN LEAVES*

Mg level (μmole)	EDDHA (cpm/aliquot)		Chelate/no chelate ratio
	None	EDDHA, 1 μmole	
0 (endogenous only)	161	224	1.4
1	204	680	3.3
2	311	917	3.0
5	956	2990	3.1
10	1050	3940	3.7
20	1110	3940	3.5
30	1460	4210	3.0

* *See* under *Experimental Methods* for general procedure.

R5P systems more than the PEP. All these effects indicate differences in the nature of the 2 reactions for CO_2 fixation.

EDDHA increased the PEP reaction more consistently in preparations from etiolated plants than in those from preparations with green plants.

Limiting Mg decreased the R5P system much more than the PEP system. It decreased C^{14} fixed with PEP when EDDHA was added and resulted in a smaller increase with R5P when EDDHA was added, as against the results of treatments with adequate Mg.

HEEDTA inhibition of both the carboxylation reactions was overcome only slightly by the addition of heavy metals to the reaction, indicating that HE-

EDTA toxicity probably is not the result of chelation. Double-reciprocal plots for the R5P system with R5P, ATP, and HCO_3^- each as substrates indicated noncompetitive inhibition of HEEDTA.

Acknowledgment

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THE ROLE OF METALS IN THE ACTIVATION OF MUSCLE PHOSPHORYLASE

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Skeletal muscle phosphorylase exists in two forms: phosphorylase *b*, which requires adenylic acid (AMP) for activity, and phosphorylase *a*, which is active in the absence of AMP.[1-5] Because of the low concentration of AMP in muscle tissue, phosphorylase *b* has been considered a physiologically inactive form of the enzyme[5] and would be analogous to the inactive phosphorylase of liver[6] in this respect.

Freshly prepared rabbit muscle extracts contain most of their phosphorylase in the *b* form.[7] When such extracts are incubated for a brief period of time in the presence of certain divalent metals, phosphorylase *b* is converted to phosphorylase *a*, provided the extracts contain sufficient ATP.[8] This is illustrated in TABLE 1. The effect of added metals is rapid, and it is possible to obtain sufficient Ca^{++} ions to effect a phosphorylase *b* to *a* conversion simply by filtering a fresh muscle extract through unwashed filter paper.[8] Ethylenediaminetetraacetate (EDTA) effectively blocks the metal effect and hence the conversion.

Role of Metals in the Activation of Phosphorylase Kinase

The action of metals in causing the activation of phosphorylase in extracts is indirect and can be understood only in terms of the enzymatic reactions that are involved in the interconversion of the two forms of phosphorylase. The formation of phosphorylase *a* from phosphorylase *b* occurs according to the following equation:[10]

$$2 \text{ phosphorylase } b + 4 \text{ ATP} \xrightarrow{Mg^{++}(Mn^{++})} \text{phosphorylase } a + 4 \text{ ADP} \quad (1)$$

The reaction is catalyzed by the enzyme, phosphorylase kinase. The 4 moles of phosphate introduced are bound to a serine residue in the enzyme at a site containing the following sequence of amino acids:[11]

$$\ldots \text{Lys.Glu-}NH_2\text{.Ileu.P-Ser.Val.Arg.} \ldots$$

The conversion of phosphorylase *a* to phosphorylase *b* is a hydrolytic reaction having the equation:[12,13]

$$\text{phosphorylase } a + 4\ H_2O \rightarrow 2 \text{ phosphorylase } b + 4 \text{ inorganic phosphates} \quad (2)$$

The latter reaction* is catalyzed by phosphorylase phosphatase, formerly called PR enzyme.[2] In rabbit muscle extract, reaction 2 does not occur to any considerable extent, presumably due to the inhibition of phosphorylase phosphatase by anionic components present in the extract. Reaction 1 does not occur spontaneously in extracts for reasons that will be discussed below.

* The change in molecular weight from 500,000 to 250,000 (which occurs in the phosphorylase *a* to *b* reaction) was first observed by Keller and Cori.[14]

Phosphorylase kinase, as extracted from skeletal muscle, is in a form that is inactive at *p*H 6.8 or below but is partially active when assayed at higher *p*H values.[15] Since muscle extracts have a *p*H of about 6.5, the kinase is inactive, and no conversion of phosphorylase *b* to *a* occurs even though the required components, ATP and Mg, are present. For the reaction to occur, the kinase must be activated, and it is this effect that is brought about by adding divalent metals other than Mg to the extract (TABLE 2). Among the metals tested in

TABLE 1

THE CONVERSION OF PHOSPHORYLASE *B* TO PHOSPHORYLASE *A* IN MUSCLE EXTRACTS

Portions of Muscle Extract (1.0 ml.) Obtained by 2 Successive Extractions of Ground Rabbit Skeletal Muscle with Equal Weights of Cold Water Were Brought to a Final Volume of 1.2 ml. with Water or Additions, as Shown. The Mixtures Were Incubated for 20 Min. at 30° C. and Assayed for Phosphorylases *b* and *a*.

Addition*	Phosphorylase *b* (per cent)	Phosphorylase *a* (per cent)
Control	92	8
K^+	88	12
Mg^{++}	86	14
Ca^{++}	0	100
Sr^{++}	0	100
Mn^{++}	0	100
Ni^{++}	92	8

* The salts added were acetates or chlorides and were present at a final concentration of 1×10^{-3} *M* during the incubation. In this experiment each incubation mixture was fortified with added ATP to a final concentration of 1×10^{-3} *M*.

TABLE 2

ROLE OF Ca^{++} IN THE ACTIVATION OF PHOSPHORYLASE KINASE

Muscle Extract at *p*H 6.5 Was Incubated for 15 Min. in the Presence and Absence of Calcium Acetate Added to a Final Concentration of 1×10^{-3} *M*. Phosphorylase Kinase Activities Were Determined at a Final Dilution of 1 to 1200 at Several *p*H Values, as Described.[15]

Addition	Phosphorylase kinase activity at			
	*p*H 6.5 (U./ml.)	*p*H 7 (U./ml.)	*p*H 7.5 (U./ml.)	*p*H 8.5 (U./ml.)
None	0	100	3,000	18,000
$Ca(Ac)_2$	10,000	13,000	17,000	20,000

the activation of phosphorylase kinase, Ca is the most effective, Mn is poorly effective, and Mg has no effect. In an extract, endogenous phosphorylase *b* is converted to phosphorylase *a* when Mn^{++} ions are added (TABLE 1), but this is equivalent to carrying out a kinase assay without any dilution of the extract, and under these conditions it suffices that the kinase be activated to only a slight degree to catalyze the reaction. It will be noted in TABLE 2 that kinase activity tests are ordinarily performed at dilutions of muscle extract greater than 1 to 1000.

As may be seen in TABLE 2, the activation of phosphorylase kinase by added Ca^{++} ions results in the formation of enzyme, which possesses half of its maximal

activity at *p*H 6.5, whereas previously it was completely inactive at this *p*H. The mechanism of the activation is unknown, but it would appear to be independent of any low molecular weight nonprotein components in the muscle extract, inasmuch as the inactive kinase has been purified approximately tenfold by acid precipitation followed by extensive dialysis without loss of the Ca-activating effect. The activation of phosphorylase kinase by Ca^{++} ions is not reversed by dialyzing the activated enzyme against EDTA at *p*H 7, although the activation can be blocked by EDTA if this component is added before the calcium salt. Studies by William L. Meyer in this laboratory have shown that a separate protein component appears to be required for the activation of kinase by Ca^{++} ions.

An entirely different action of Ca^{++} ions illustrates how complicated a metal ion effect can be in a given enzyme system. Although incubation of inactive

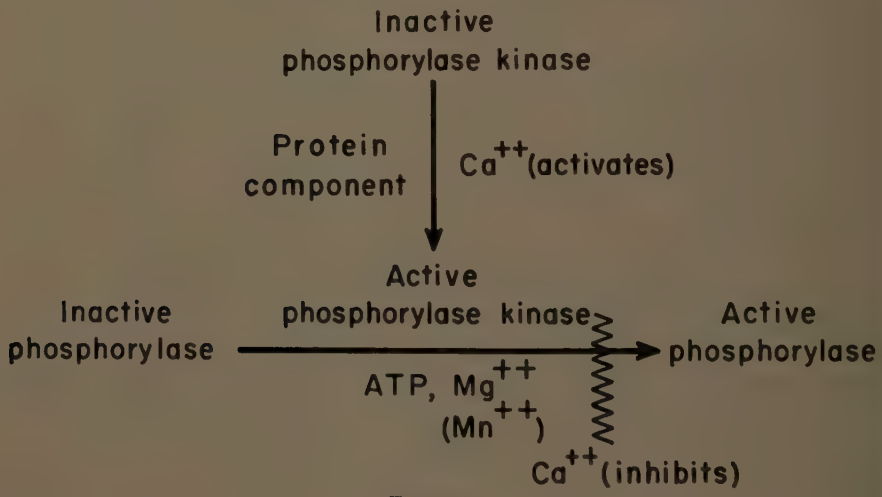

FIGURE 1.

kinase with Ca serves to activate the enzyme as described above, this metal is actually an inhibitor of the phosphorylase kinase reaction itself, since it competes with Mg^{++} ions required in the reactions.[15] The dual role of Ca can be demonstrated readily by carrying out the activation reaction at a sufficient concentration of kinase so that a large dilution is required before this enzyme is introduced into kinase reaction mixtures with or without added Ca. These metal effects are summarized in FIGURE 1.

Activation of Phosphorylase Kinase by ATP, Mg^{++}, and Cyclic AMP

A different mechanism for the activation of phosphorylase kinase involves adenosine 3′,5′-phosphoric acid (cyclic AMP), ATP, and Mg^{++} ions.[15] In the experiment of TABLE 3, using a purified preparation of inactive phosphorylase kinase, a marked activation is obtained by incubating the enzyme with these components. The extent of activation is essentially as great as can be achieved by treating the enzyme with calcium (*see* footnote in TABLE 3). Control ex-

periments in which ATP is left out (not illustrated in TABLE 3) show no effect of cyclic AMP by itself. Mg and ATP alone will activate this type of kinase preparation, although the rate at which the activation reaction occurs is about one fifth of that obtained when cyclic AMP is included in the reaction mixture. It has not been determined whether the inactive kinase preparation may contain traces of cyclic AMP or whether some cyclic AMP may be formed from ATP under these conditions. It will be noted in TABLE 3 that the activation of the kinase is readily apparent whether the enzyme is assayed at *p*H 6.9 or at *p*H 8.2; in this way, the behavior of the purified fraction differs from that of crude muscle extracts[15] in which little activation is seen at the higher *p*H.

Sutherland and his co-workers[16,17] have shown that cyclic AMP is produced from ATP as a result of the action of epinephrine on tissues. This nucleotide in turn was shown to be responsible for the formation of active liver phosphorylase from inactive phosphorylase. The present and previously published work[15] would make it appear certain that the action of cyclic AMP is on phosphorylase

TABLE 3

ACTIVATION OF PHOSPHORYLASE KINASE WITH ATP, Mg^{++}, AND CYCLIC AMP

Inactive Phosphorylase Kinase, Purified by Acid Precipitation at *p*H 6.0 and Clarified by Centrifugation at 100,000 g to Remove More Readily Sedimentable Material, Was Incubated at 30° C. in a Reaction Mixture Containing 0.003 *M* ATP, 0.01 *M* $Mg(Ac)_2$, 1.25×10^{-4} *M* Cyclic AMP, and 0.005 *M* Cysteine. The *p*H Was 7.0 and the Concentration of Phosphorylase Kinase Was 3 mg./ml. At Intervals Samples Were Removed, Diluted, and Assayed for Phosphorylase Kinase.

*p*H of kinase assay	Phosphorylase kinase activity* in U./ml. $\times 10^{-3}$ at following times				
	0 min.	1 min.	5 min.	10 min.	20 min.
6.9	1.9	15.8	25.8	27.3	23.0
8.2	14.9	36.6	38.8	42.4	35.3

* Same fraction activated by incubation with $Ca(Ac)_2$ gave a final activity of 32.2×10^3 U./ml. at *p*H 6.9 and 39.9×10^3 U./ml. at *p*H 8.2.

kinase. Experiments carried out by D. J. Graves in this laboratory have shown only slight inhibitory effects of cyclic AMP on phosphorylase phosphatase, although the reaction was carried out at concentrations of cyclic AMP greatly exceeding those at which the nucleotide is effective in its activation of the kinase. Rall *et al.*,[18] using liver slices in which phosphorylase phosphatase was blocked with fluoride, obtained evidence several years ago that was compatible with the interpretation that epinephrine was exerting its action by stimulating the kinase system.

Possible Role of Metals in the Structure and Activity of Muscle Phosphorylase

In addition to the role of metals in the activation of phosphorylase kinase and in the phosphorylase kinase reaction, the question arises as to whether metals are involved in the structure or function of phosphorylase itself. Phosphorylase *a* with a molecular weight of approximately 500,000 dissociates into 4 subunits when the enzyme is treated with *p*-chloromercuribenzoate;[19] this dissociation can be reversed by dialysis against cysteine. Phosphorylase *b* with a molecular

weight of 250,000 is dissociated into 2 of the smaller units. As noted earlier in this paper, 4 moles of phosphate are introduced into phosphorylase in the conversion from phosphorylase *b* to *a*. Lastly, phosphorylase *a* contains 4 moles of firmly bound pyridoxal phosphate (PLP).[20,21] These findings make it possible to visualize phosphorylase *a* as having the structure illustrated diagrammatically in FIGURE 2.

The four phosphate groups introduced into phosphorylase in the *b* to *a* reaction are not involved directly in linking together the subunits of phosphorylase *a*: for example, through phosphodiester or pyrophosphate bonds. The possibility was considered, however, that these groups might participate in the binding of the subunits by chelation of a metal ion. Conversion of phosphorylase *b*

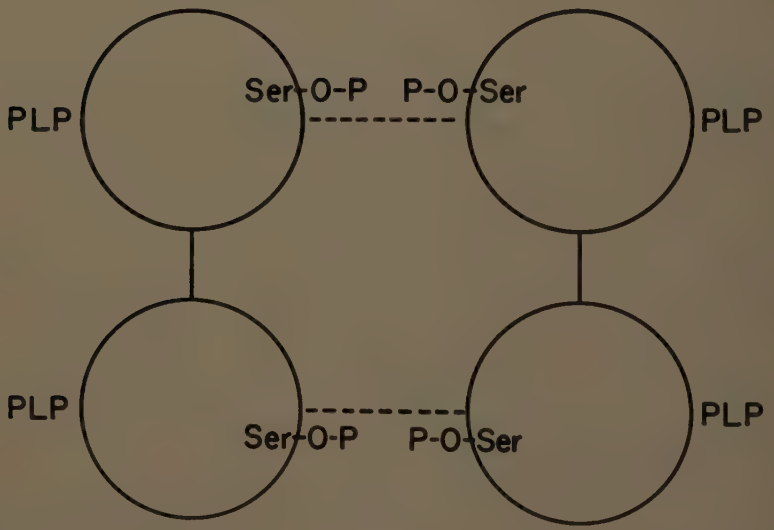

FIGURE 2.

to phosphorylase *a* was carried out using ATP and radioactive Mn (Mn^{54}), and the enzyme was isolated and crystallized; no incorporation of the isotopes was found.[10] This experiment does not eliminate the possibility that other metals might be involved, although this is unlikely in view of the stability of phosphorylase *a* toward incubation or dialysis against chelating agents.[8,19,22] Phosphorylase *b* crystallizes[21] in the presence of divalent metal ions and AMP in the form of a complex probably having the structure (phosphorylase *b*-AMP-$Me^{++})_2$. This complex is readily broken down by chelating agents.

A direct and complete metal analysis has not as yet been carried out on crystalline phosphorylase *a*; this should be done in view of the highly complex structure of this enzyme. The activity of occasional preparations of the enzyme has been found to be influenced by adding metal ions,[8] although this has not been a constant finding.

Discussion

In animal tissues the phosphorylase system of enzymes, which includes the two forms of phosphorylase, phosphorylase phosphatase, and phosphorylase kinase, together with possible ancillary enzymes that may be involved in the activation of the kinase, represents an important regulatory mechanism in carbohydrate metabolism. The system is so complex that it is not possible at this time to give a simple picture as to how it is controlled within the living cell. An investigator carrying out experiments *in vitro* with cellular materials finds himself working under conditions that are in many ways analogous to those that are faced in research on blood clotting, and a complete listing of all the protein and nonprotein factors involved in finally determining activity of the enzyme phosphorylase can successfully rival a comparable listing for the blood-clotting story.

It is of interest to speculate whether Ca^{++} ions may play some role in linking the process of muscle contraction to glycogenolysis. A release of this metal from cellular binding sites, occurring at the time of muscle contraction,[23,24] could result in the activation of phosphorylase kinase, which in turn could bring about the formation of active phosphorylase and cause glycogen breakdown. It is known[25] that muscle phosphorylase *a* is formed as a result of electric stimulation, and experiments are in progress to determine whether this is brought about through activation of phosphorylase kinase.

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PRELIMINARY STUDIES ON AN ALGAL PHOSPHORYLASE-MANGANESE CHELATE*

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The synthesis of polysaccharides in the blue-green alga *Oscillatoria princeps* is brought about by an enzyme system comprised of two enzymes, a conventional phosphorylase (P enzyme) and a branching enzyme, or transglucosidase (Q enzyme).[1,2] The P enzyme adds glucopyranose residues from glucose-1-phosphate in alpha-1:4-glucosidic linkages to a linear maltosaccharide "primer." The Q enzyme causes a scission of the linear maltodextrin so formed, and an apposition of the fragments in alpha-1:6-glucosidic linkages to the remaining linear configurations.

The polysaccharide isolated from normal (*n* strain) cultures of this alga was a polyglucoside showing a degree of branching intermediate between animal glycogen and plant amylopectin.[1,3] The characteristics of this phytoglycogen are shown in TABLE 1.

Crude aqueous extracts of this alga caused the synthesis of this polyglucoside from the Cori ester *in vitro*. A mutant (*LTV* strain) of this alga was reported that formed a polyglucoside considerably less branched than that described.[2,3] Extracts prepared from artificially maintained cultures of this (*LTV* strain caused the synthesis, from buffered substrates of glucose-1-phosphate, of a polyglucoside with the properties summarized in TABLE 1.

Ammonium sulfate fractionation of the extracts from these two strains of *Oscillatoria* and subsequent physicochemical studies of the isolated enzymes showed no essential differences between them.[4,5] However, it was noted that mixtures of the P and Q enzymes were obtained that caused a wide spectrum of branched polyglucosides to result from the action on the Cori ester.

With the purified enzymes it was possible to cause the synthesis of branched or unbranched polyglucosides by varying the relative amounts of P and Q enzymes in the mixtures. This had been reported previously for mixtures of the phosphorylase and Q enzyme of the potato[6,7] and postulated as a possible explanation of the various degrees of branching reported in rabbit liver glycogen.[8] It had also been observed that, during the paper electrophoresis of the two enzymes in potato, branched and unbranched sugars were obtained, depending upon the completeness of the separation and the extent of overlapping of the enzymes on the paper.[9]

In general, the immediate character of the polyglucoside synthesized by mixtures of the P and Q enzymes appeared to be determined by the ratio of the two enzymes in the mixture. In *O. princeps* this Q/P ratio operated such that, when there was a large excess of P enzyme present in the mixture, long, relatively unbranched polyglucosides were synthesized from the Cori ester; when the P enzyme component was low, highly branched, glycogenlike polymers resulted from the action of the mixture.[10,11] These observations tended to sub-

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stantiate the hypothesis of random scission of linear maltodextrins by Q enzyme.[6,7]

Quantitative electrophoretic studies of these enzymes in the *n* and *LTV* strains of this alga showed that the ratios of the two enzymes in the crude extracts were about the same, yet two differently branched sugars were formed by these extracts.[4,5,12] The only difference noted during these studies was the presence of substantial quantities of ninhydrin-positive material (in zones devoid of enzyme activity) in the *n* strain extracts and its absence in the *LTV* strain extracts.[4] Similar material has been encountered in high-speed centrifugal chromatography of extracts from various Cyanophyceae, including the *n* and *LTV* strains of *O. princeps*.[13]

Because of the possible interference and deactivation due to iron contamination,[7] ethylenediaminetetraacetate salts (EDTA) were incorporated into the procedures during the preparation of P and Q enzymes. It was noted, however, that inactive P enzyme preparations resulted from their use during fractionation of the extracts. Further detailed studies revealed the fact that if EDTA was

Table 1
Properties of *Oscillatoria* Polyglucosides

	n Strain*	*LTV* strain*
Solubility (water)	Soluble[1,3]	Retrogrades[2,3]
Unit	Glucose[1]	Glucose[2]
Iodine color	Violet[1,3]	Blue[2,3]
Absorption maximum of iodine complex	550 mμ[1-3]	610 mμ[2,3]
End group assay value	19 to 21[3]	25 to 29[3]

* Superscript numbers are bibliographic references.

added to *LTV* strain crude extracts, branched polyglucosides were formed that were closely akin to those formed by untreated *n* strain extracts.[14]

Dialysis of active P enzyme extracts against 23.5 per cent solutions of the polymer polyvinylpyrrolidone (PVP)* also yielded inactive phosphorylase preparations[5] (J. F. Fredrick; in preparation). This inactivation of the P enzyme did not occur when dialysis against cold water or cold glycerophosphate buffer was carried out.[1] Hence, it was decided to study the effect of various metallic ions for possible activation of this algal P enzyme.

Preparations of *O. princeps* P enzyme that had been dialyzed against PVP for 24 hours showed approximately one third the activity of water-dialyzed preparations. Full activity could be restored when manganese acetate was added to the dialyzed P enzyme preparation prior to incubation in the reaction mixture. Calcium and magnesium ions were ineffective.

Attempts at purification of this algal enzyme with the use of ion-exchange resins also resulted in partially inactivated preparations of P enzyme. Completely inactive P enzyme preparations could be prepared by dialysis against PVP followed by treatment with a cation exchange resin.

* NP K-30, Antara Chemicals, New York, N. Y.

Experimental

Extracts were prepared from healthy cultures of the *n* strain of *O. princeps*.[1] These extracts were fractionated with ammonium sulfate as previously described,[15] and the P enzyme was dissolved in glycerophosphate buffer at *p*H 6.8. A portion of this enzyme solution was dialyzed in Visking against 6 changes of 23.5 per cent PVP for 24 hours at 6° C. The solution was diluted with buffer to a concentration of approximately 10 mg. of protein per milliliter. This preparation was treated with Dowex A-1 resin (50 to 100 mesh), which had been converted to the sodium form[16] and further prepared as indicated below.

The sodium form of Dowex A-1 was washed with cold 0.030 *M* sodium beta-glycerophosphate until completely free of "color throw." The resin was stored wet in the refrigerator until just before use. Fifteen ml. of this cold resin slurry was shaken with 50 ml. of the PVP-dialyzed P enzyme preparation at 6° C. for 1 hour. The resin was separated by filtration through cotton,* and the resin-treated P enzyme filtrate so obtained was used for these studies.

Owing to dilution and adsorption of part of the protein on the resin, the resulting enzyme solution contained 7.5 mg. of protein per milliliter. In all experiments a total protein concentration of 15 mg. was used in a total volume of 10 ml. of reaction mixture. The reaction mixture contained 0.055 *M* glucose-1-phosphate as the dipotassium salt, 0.1 per cent amylose (previously solubilized by sodium hydroxide and then neutralized with hydrochloric acid) in 0.025 *M* sodium beta-glycerophosphate —0.020 *M* tris(hydroxymethyl) amino methane buffer at *p*H 7.1. Incubation was at 30° C.

Adenosine-5-phosphate was omitted after preliminary studies (Fredrick; in preparation) had indicated that it had not appreciably affected polyglucoside synthesis by algal P enzyme. Adenosine-5-phosphate has not been considered essential for the activity of plant phosphorylases.[7,18]

Where metallic salts were added, they were added directly to the resin-treated enzyme preparation at room temperature (23° C.) 1 hour prior to addition of the enzyme to the reaction mixture. This incubation period was found necessary for maximal effect.[19]

The liberation of inorganic phosphate from the Cori ester and the concurrent lengthening of the amylose "primer" chain (as measured by glucose determinations on the precipitated polysaccharide[1,15]) were used to follow P enzyme action in the direction of polyglucoside synthesis. Comparisons were made with undialyzed and nonresin-treated P enzyme in identical reaction mixtures at the same time intervals. The time intervals selected for the comparison data were those at which 20 per cent conversion of glucose-1-phosphate to phytopolyglucoside was achieved with untreated P enzyme. At these points the reaction is proportional to the P enzyme concentration.[1,15]

Results

There was a progressive increase in phosphorylase activity in the direction of polysaccharide synthesis by the dialyzed resin-treated enzyme with increas-

* Filter paper may contribute metallic ions.[17]

ing concentrations of manganous ion. As shown in FIGURE 1, low concentrations of manganous ion caused slight increases in polyglucoside synthesis by P enzyme. However, as the concentration of Mn^{++} ions increased above 10^{-5} M, there was a rapid, almost first-order increase in the polyglucoside-synthesizing activity of the resin-treated enzyme preparations. The optimal concentration was at about 10^{-2} M of manganous ion, at which point the resin-treated P enzyme preparations exhibited full activity, in contrast to the untreated P enzyme.

The results with magnesium and calcium were negative. The inhibitory effect of the ferric ion was confirmed.[15]

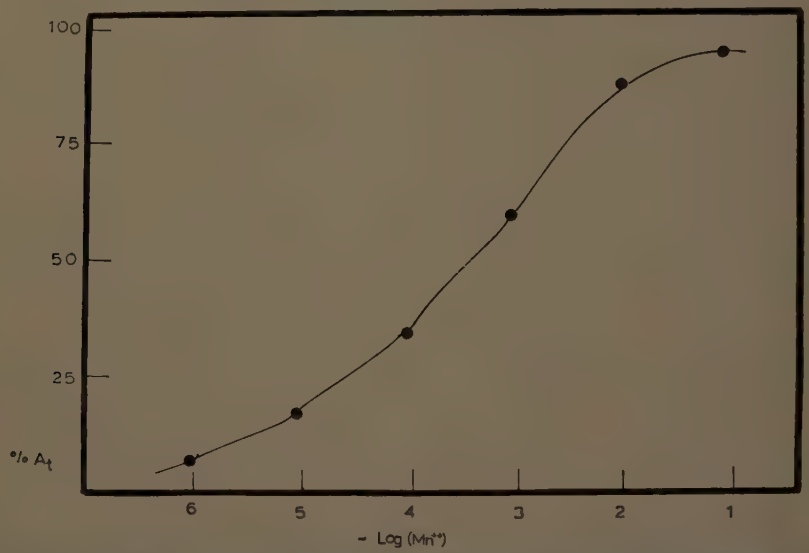

FIGURE 1. Effect of manganous ion in restoring polyglucoside-synthesizing action to resin-inactivated algal P enzyme. The concentrations of manganous ion, $-\log (Mn^{++})$, are shown on the abscissa, and the per cent activity, A_t, as compared with that of untreated enzyme, is shown on the ordinate.

Discussion

It is possible to use the curve of FIGURE 1 for a rough estimate of the strength of the P enzyme-manganese bond. For example, at the point of 50 per cent (of total) activity, the manganese-binding positions on the enzyme molecule may be assumed to be half filled, and hence

$$(\text{P protein}) = (\text{P enzyme}).$$

Assuming the activation reaction to be

$$\text{P protein (inactive)} + Mn^{++} \rightleftharpoons \text{P enzyme (active)}$$

and applying the mass action law

$$K = (\text{P enzyme})/(\text{P protein})(Mn^{++})$$

with the understanding that under the conditions described the expressions in

parentheses apply not to absolute concentrations of protein and enzyme, but to molar concentration of ligand positions of the P protein that combine with manganese and to molar concentration of manganese ions bound in the P enzyme. Then, since

$$(\text{P protein}) = (\text{P enzyme}),$$

the K for the reaction becomes

$$K = 1/(Mn^{++})$$

and

$$\log K = \log 1 - \log (Mn^{++})$$

or [19,20]

$$\log K = -\log (Mn^{++}),$$

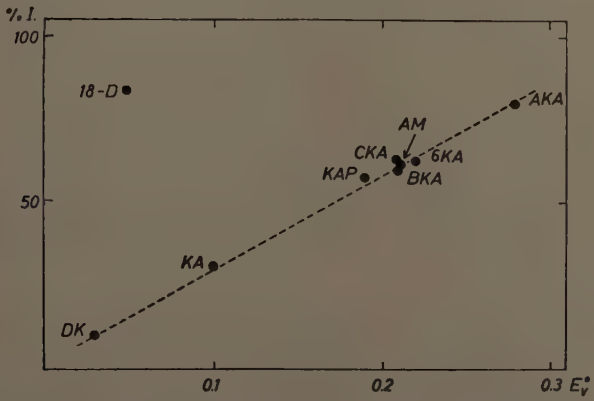

FIGURE 2. Inhibition of algal P enzyme as a function of chelate stability. The relative stabilities of the kojic acid derivatives (determined by redox potentials[21]) are shown on the abscissa, and the per cent inhibition of polyglucoside synthesis by algal P enzyme is indicated on the ordinate. The abbreviations for the kojic acid derivatives are as follows: *DK*, 6,6′-dikojyl methane; 18-D, a quaternary derivative of kojic acid; *KA*, kojic acid; *KAP*, 2-hydroxymethyl-5-hydroxy-γ-pyridone; *BKA*, bromokojic acid; *CKA*, chlorokojic acid; *AM*, 2-methyl-5-hydroxy-γ-pyrone; *6KA*, 2,6-dihydroxymethyl-5-hydroxy-γ-pyrone; *AKA*, 2-acetoxymethyl-5-hydroxy-γ-pyrone. Reproduced by permission of *Physiologia Plantarum*.[21]

so that, reading directly from the curve (FIGURE 1) at the point of 50 per cent activity, the log K of the P enzyme-manganese chelate is approximately 3.2.

This stability constant is of the right order when it is considered in the light of the previous work on the effect of synthetic chelating agents on this algal P enzyme.[14,21] For example, it was found that kojic acid and its derivatives inhibited *O. princeps* P enzyme and that this degree of inhibition was related directly to the stability of the kojic acid derivative-metal chelate formed.[21] This is shown in FIGURE 2.

Note that the 6,6′-dikojyl methane derivative had the least inhibitory effect on this P enzyme and also that its manganese chelate exhibited a relatively lower order of stability than did the other kojic acid derivatives. Job analysis had

indicated that this derivative formed chelates with the first group transition metals that were affected by steric hindrance[22] (FIGURE 3).

It appears that any chelating agent capable of forming a manganous chelate whose log K is greater than that of the P enzyme inhibits the P enzyme's activity, probably by causing a withdrawal of the ion from the enzyme (TABLE 2). Even 6,6′-dikojyl methane with a log K certainly similar to, if not identical with, that of the algal P enzyme can partially inhibit the polyglucoside-

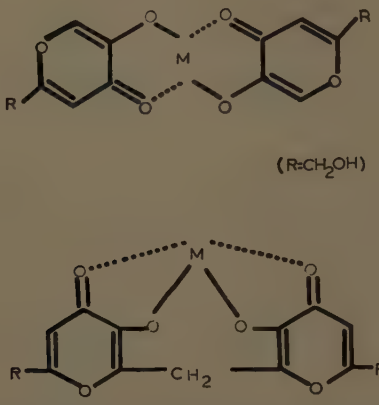

FIGURE 3. Structural diagrams of two types of chelates formed by kojic acid compound and first group transition metals. The stability of the kojic acid chelate (*top*) is much greater than that of the 6,6′-dikojyl methane derivative (*bottom*).

TABLE 2
STABILITY CONSTANTS OF MANGANESE CHELATES

Chelate	log K*
EDTA-manganese	13.4[21]
Dowex A-1-manganese	4.4[24]
Dikojyl methane-manganese	3.5[23]
P enzyme-manganese	3.2

* Superscript numbers are bibliographic references.

synthesizing action of the enzyme. A possible method of action of the P enzyme as it involves polyglucoside synthesis is shown in FIGURE 4.

Reference has been made to the ninhydrin-positive material present in the crude extracts of *n* strain of *O. princeps* and its absence in *LTV* strain extracts. This material exhibited fluorescence under ultraviolet light *after* the paper chromatograms had been heated.[13] Further paper chromatographic studies strongly indicate that this material may be of amino acid structure. Its presence in only *n* strain extracts suggests its participation in the possible "demetallization" of P enzyme in these extracts, and hence a *modus operandi* for control of the Q/P ratio. Its action in deactivating the P enzyme would be tantamount to a decrease of the P enzyme component of this ratio.

Conclusions

The synthesis of polysaccharides by the blue-green alga *Oscillatoria princeps* is due to a conventional phosphorylase and to a transglucosidase or Q enzyme. The degree of branching in the polysaccharide is controlled by the ratio (Q/P) of each enzyme present in a mixture. Manganese, as the manganous ion, forms a chelate with the phosphorylase that is essential for polyglucoside synthesis. Demetallization of the phosphorylase with ion-exchange resins results in an inactive preparation whose activity is restored by manganous ions. Synthetic and natural chelating agents that can cause an inactivation of the phosphory-

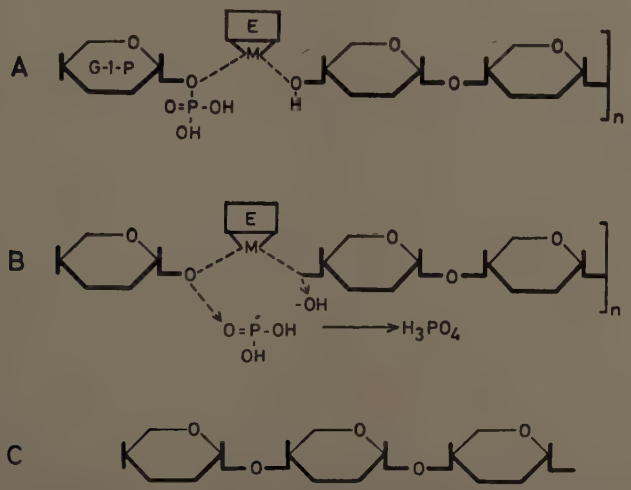

FIGURE 4. Possible mode of action of *Oscillatoria* phosphorylase-manganese chelate. A shows the establishment of a 3-unit chelate between the P enzyme (E) and its manganese (M), glucose-1-phosphate (G-1-P) and the linear maltosaccharide "primer" of "n" glucose residues. B shows the release of the H_2PO_3 radical by the glucose-1-phosphate as a result of the chelate binding and also the simultaneous release of the hydroxyl group of the 4-carbon of the end residue of the "primer." The recombination of these two radicals yields phosphoric acid. C shows the resulting maltodextrin with the new alpha-1:4-glucosidic bond established. This new dextrin now contains "n + 1" glucose residues, etc. Reproduced by permission of *Physiologia Plantarum*.[21]

lase can thereby control the Q/P ratio. It is suggested that this is a possible physiological mechanism, present in *n* strains of the alga and absent in *LTV* strains, for the *in vivo* determination of the degree of branching of the polyglucosides in this group.

Acknowledgments

Thanks are due to the Dow Chemical Company, Midland, Michigan for making available the Dowex A-1 resin used in these studies and to W. J. Whelan of the Lister Institute, London, England, for his valuable suggestions.

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THE INFLUENCE OF CHELATION IN DETERMINING THE REACTIVITY OF THE IRON IN HEMOPROTEINS, AND THE COBALT IN VITAMIN B_{12} DERIVATIVES*

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In the hemoproteins and in vitamin B_{12} and its derivatives the iron and cobalt are already bonded in complicated chelate structures that are remarkably stable. There is good evidence that this stability has its origin in extremely favorable free energies of formation, and that even under conditions in which dissociation would be favored, such as strong acidity, the liberation of the metal ion is a very slow process. For these reasons there are scarcely any quantitative data on the fundamental reactions by which the metal ions are incorporated in these molecules; thus concerning chelation phenomena the general topic remaining is the influence that this chelation has on the reactivity of the metal ions.

From the point of view of the biochemist this is the more important topic because these particular structures enable the metal complexes to take part in highly specific chemical reactions utilized by the biological systems, reactions that occur to a far lesser extent, if at all, with the aquo ions or with simpler coordination complexes. Hemoglobin, peroxidase, and catalase furnish striking examples: the first, in its Fe^{II} oxidation state, undergoes a rapid and reversible combination with molecular oxygen, the equilibrium constant having the very favorable value of about 10^6 M^{-1}; the latter two, in their Fe^{III} oxidation states, react with strong oxidizing agents to give higher oxidation states, 1 and 2 equiv. above the Fe^{III} state. However, from the point of view of the chemist, the reactions that these biologically important iron and cobalt complexes have in common with the aquo ions and simpler complexes are no less significant; moreover, a study of these common reactions, through the various comparisons that they afford, may eventually lead to a better understanding of the special structural factors determining the highly specific reactions.

This paper deals with some of these common reactions. It is divided into the following sections.

(1) An outline of the main structural features of the chelation present in hemoproteins and in vitamin B_{12} and its derivatives.

(2) A comparison of thermodynamic data for corresponding reactions of hemoglobin and the Fe^{++} and Fe^{+++} aquo ions, in an attempt to define the nature and extent of the influence exerted by the chelation.

(3) A discussion of ligand replacement reactions of vitamin B_{12} and its derivatives. This includes an experimental study of the reactions of various ligands with factor B, extending the scope of previous comparisons made

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between aquocobalamin and the iron porphyrins. In addition, approximate values have been obtained for the stability constants associated with the bonding of the cobalt to the benzimidazole and cyanide groups in vitamin B_{12} and to the cyanide groups in factor B and the dicyano factor.

(4) Comments on the stabilization of the oxidation states of the iron and cobalt by the chelate ring systems.

Main Features of the Chelate Structures

The structure of ferriprotoporphyrin IX, the prosthetic group of many hemoproteins, is shown in FIGURE 1,[1,2] and that of vitamin B_{12} in FIGURE 2.[3,4] The chelation within the compact, more or less planar, heterocyclic ring sys-

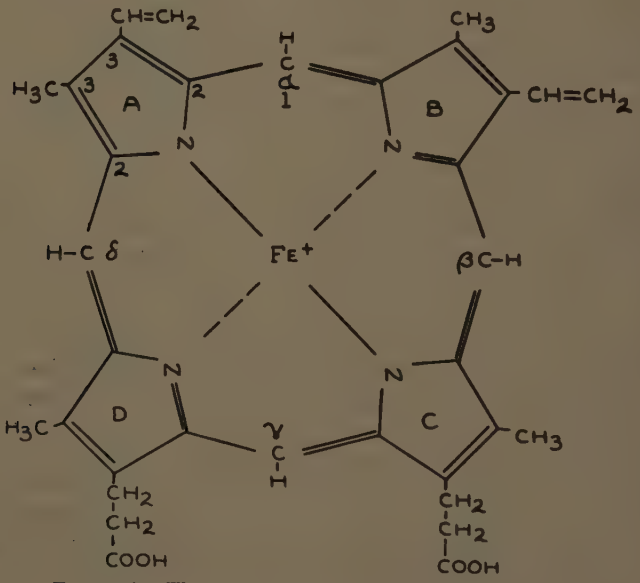


FIGURE 1. The structure of ferriprotoporphyrin IX.[1,2]

tems, in which 4 nitrogen atoms are bonded to the metal ion, has the great thermodynamic and kinetic stability referred to and, were it not for weaker, more labile bonding to the groups occupying the fifth and sixth (that is, *trans*) coordination positions of the octahedral complex, these iron and cobalt compounds would engage in very few ligand replacement reactions.

In vitamin B_{12} these positions are occupied by a cyanide group and by the glyoxalinium N atom of the 5:6-dimethylbenzimidazole moiety of the nucleotide, which is joined to the rest of the ring system via a phosphate ester and an amide linkage, as indicated in FIGURE 2. Three derivatives of vitamin B_{12}, which has been given the systematic name cyanocobalamin, are dealt with in reactions described later. In aquocobalamin, as the name suggests, the cyanide group is removed, and it is assumed that a water molecule takes its place.[5] Factor B, prepared by acid hydrolysis, lacks the entire nucleotide, the side chain terminating at the amino alcohol residue, but the cyanide group is re-

tained: in the diaquo factor the cyanide group is also absent.[6] In these factors, 1 or 2 water molecules, respectively, presumably complete the coordination shell. Since the main chelate ring system remains intact, the reactions of the

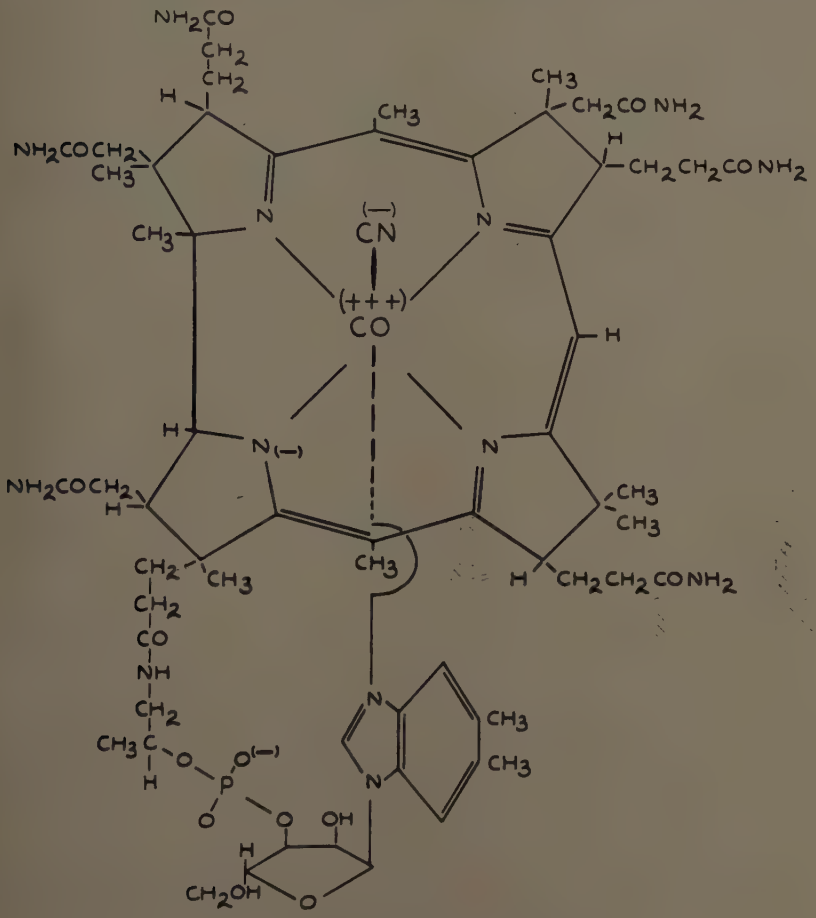
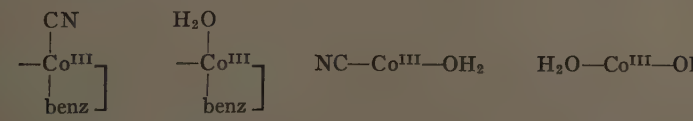


FIGURE 2. The structure of vitamin B_{12}.[3,4]

cobalt in these compounds may be represented conveniently by the formulas



(cyano-cobalamin) (aquo-cobalamin) (factor B) (diaquofactor)

where the plane containing the heterocyclic ring is understood to be a horizontal plane perpendicular to this page in the first and second formulas, and a vertical plane perpendicular to the page in the third and fourth.

In the hemoproteins, the groups that occupy the fifth and sixth coordination positions have not yet been identified; nevertheless, certain generalizations may be made and, on the basis of spectral evidence, certain types of bonding may be ruled out in particular cases. There seems little question that in all the hemoproteins there is at least one bond between the iron and a group in the protein, for otherwise it would be extremely difficult to account for the specificity of hemoprotein reactions compared to those of ferrous and ferric porphyrins, and for many significant differences in the absorption spectra and magnetic susceptibilities.[7] On this assumption, three kinds of structure may be envisaged, as shown in FIGURE 3. In *B* the porphyrin ring lies flat on the surface of the protein. In *A* another part of the protein molecule coils around near the exposed side of the porphyrin, shielding the iron from attack by ligands, but still sufficiently far away that a second iron—protein bond cannot

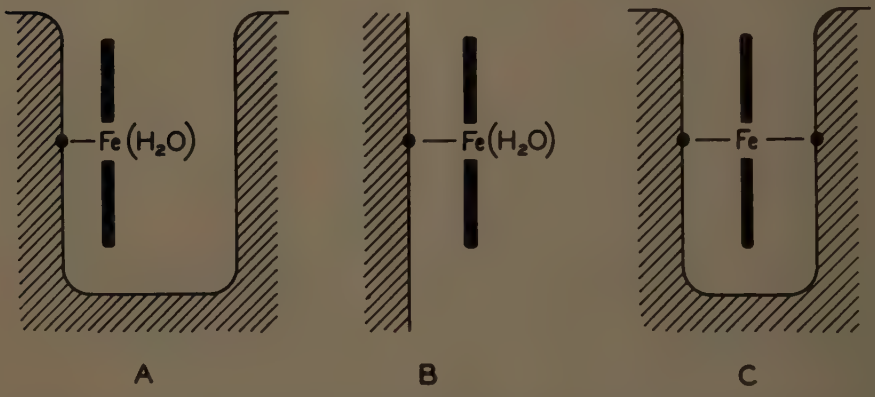


FIGURE 3. Generalized structures for hemoproteins.[8] (*A*) Crevice structure with one Fe—protein bond. (*B*) Porphyrin lies flat on surface of protein. (*C*) Crevice structure with two Fe—protein bonds. The plane of the porphyrin ring is understood to be perpendicular to this page, and bonding via the side chains of the porphyrin has been omitted. Reproduced by permission of the National Academy of Sciences.

form. With such structures it is assumed that a water molecule completes the coordination shell as before. In *C* there are two Fe—protein bonds, and the iron porphyrin may be regarded as bound within a "crevice" in the protein. For brevity, *A* and *C* may be termed one-bond and two-bond crevice structures respectively. Since the chelation within the porphyrin ring again remains intact, the reactions of ferrihemoproteins having either the structure of *A* or of *B* may be represented by the formula $\text{Prot—Fe}^{\text{III}}\text{—OH}_2$, and those with the structure of *C* by formulas of the type $\overline{\text{Prot—Fe}^{\text{III}}\text{—X}}$, where X is the group in the protein replaced by the ligand. The reactions of the ferrohemoproteins also may be shown by the formulas $\text{Prot—Fe}^{\text{II}}\text{—OH}_2$ and $\overline{\text{Prot—Fe}^{\text{II}}\text{—X}}$.

In cytochrome *c*, spectroscopic evidence supports a two-bond crevice structure with the iron linked to nitrogenous base groups:[9] protein degradation studies[10,11] and the *p*H variation of the stability constant for the formation of its azide complex[12] indicate that one of these groups is a histidine residue.

Furthermore, analytic studies have revealed that the porphyrin is also bonded to the protein, via thioether linkages formed between the vinyl groups and cysteine residues.[13-15] X-ray analysis has shown that in myoglobin the iron porphyrin is bound in a shallow one-bond crevice structure,[16] and the same configuration probably is present in hemoglobin (M. Perutz; personal communication, October 1959). Histidine has been implicated as the group joined to the iron.[17] In peroxidase and catalase the anomalous *p*H variation of the stability constants for complex formation may be explained by a two-bond crevice structure in which one of the bonds is especially labile, involving a group that has a high proton affinity but is not a nitrogenous base.[8] A more detailed discussion of the groups that might be attached to the iron in these and other hemoproteins is beyond the scope of this paper; reference may be made to several recent articles.[7,8,18] However, this very brief outline brings out the points of structural similarity between the hemoproteins and the cobalamin compounds. In some respects these cobalt complexes may be regarded as "hemoprotein models," the amino alcohol-nucleotide moiety having taken the place of the protein.

It may now be seen that a second kind of chelation is present. Besides the chelation within the more or less planar heterocyclic ring systems, the bonding of the nucleotide in cyano- and aquocobalamin and the bonding of the nitrogenous base groups in cytochrome *c* result in the formation of very large chelate rings which, for example, in the case of the cobalamins, contain 19 atoms. If there is some form of attachment between the porphyrin side chains and the protein in myoglobin and hemoglobin[16] and if peroxidase and catalase have some kind of two-bond crevice structure,[8] then similar large chelate rings would also be present in these compounds. As yet there is no evidence to suggest that this subsidiary chelation makes any special contribution to the reactivity of the metal ions other than in filling one or both of the remaining coordination positions. However, since the bonding is an intramolecular process, its dissociation will not show the concentration dependence that characterizes the fission of intermolecular complexes (that is, $AB \rightleftharpoons A + B$).

Comparison of Thermodynamic Data for Corresponding Reactions of Hemoglobins and the Fe^{++} and Fe^{+++} Aquo Ions

Certain biological functions of hemoproteins depend on the property of binding a ligand far more favorably than does the aquo ion or its simpler coordination complexes. For example, there is ample kinetic evidence to show that the Fe^{++} aquo ion forms a complex with molecular oxygen,[19] yet it is clear that the equilibrium constant for its formation is at least 10^8 times smaller than that for hemoglobin. It is of interest therefore to enquire whether this more favored ligand bonding, a direct consequence of the chelate structure present in the hemoprotein, originates in the heat or entropy terms that make up the standard free energy change, according to the thermodynamic equation, $\Delta F^\circ = \Delta H^\circ - T\Delta S^\circ$.

A study of the data for corresponding reactions of the Fe^{II} and Fe^{III} oxidation states of the hemoglobins and the Fe^{++} and Fe^{+++} aquo ions that were available some years ago showed that the hemoprotein does not always have the higher affinity for the ligand.[20] In the case of the Fe^{III} derivatives, where

the hemoglobin complexes have a high magnetic susceptibility, for example, with F^-, CNS^-, and OH^-, the ΔF° values for the Fe^{+++} aquo ion are systematically more favorable. On the other hand, in the case of the Fe^{III} derivatives where the hemoglobin complexes have low magnetic susceptibilities, for example with N_3^- and CN^-, and in the case of the Fe^{II} derivatives, the ΔF° values for hemoglobin are the more favorable. But it appeared that ΔH° was invariably far more favorable for the hemoglobin reactions, and that ΔS° was invariably far less favorable. In a further discussion of the entropy changes, two criteria were employed. Each made use of the quantity $(\bar{S}^\circ_{ML} - \bar{S}_M^\circ)$, namely, the difference between the partial molal entropies of the complex ML and its parent metal compound M. This quantity readily can be calculated from the expression

$$\Delta S^\circ = \bar{S}^\circ_{ML} - \bar{S}_M^\circ - \bar{S}_L^\circ \qquad (1)$$

where $\bar{S}_L^\circ$ is the partial molal entropy of the ligand.

For neutral monodentate ligands, an examination of the data for many simple coordination complexes gave the general rule[21]

$$(\bar{S}^\circ_{ML} - \bar{S}_M^\circ) / \bar{S}_L^\circ \approx 0.9. \qquad (2)$$

The partial molar entropies of neutral ligands are all positive and range upward from about 25 entropy units (eu), increasing with increasing molecular size and complexity. This rule thus implies that, when a ligand is bonded, the partial molal entropy of the complex is increased above that of the parent compound by about 0.9 $\bar{S}_L^\circ$. For example, for NH_3, one of the simplest ligands, $\bar{S}^\circ = 26.3$ eu, and the increase is about 23 eu. Data for ferromyoglobin's* combining with O_2 and with CO gave, however, small negative values for $(\bar{S}^\circ_{ML} - \bar{S}_M^\circ)$. The fact that $\bar{S}^\circ$ for O_2 and for CO has the values 25.7 and 25.5 eu, respectively, suggested that an abnormally low entropy change characterizes the formation of complexes between neutral ligands and the Fe^{II} oxidation state of the hemoprotein.

For the bonding of anionic ligands, the quantity $(\bar{S}^\circ_{ML} - \bar{S}_M^\circ)$ was used in a different way. With the "ion-pair" complexes formed between the Fe^{+++} aquo ion and OH^-, F^-, Cl^-, Br^-, CNS^-, and N_3^-, it was found that

$$(\bar{S}^\circ_{ML} - \bar{S}_M^\circ)$$

was approximately constant, independent of the ligand, and roughly equal to +43 eu, the difference between the partial molal entropies of the Fe^{++} and Fe^{+++} aquo ions themselves. This was taken as an indication that desolvation, resulting from partial charge cancellation, is the dominant feature.[22] On the other hand, for the OH^-, F^-, and CN^- complexes of ferrimyoglobin, $(\bar{S}^\circ_{ML} - \bar{S}_M^\circ)$ was found to be about zero, so it was concluded that an abnormally low entropy change also characterizes the formation of complexes between anionic ligands and the Fe^{III} oxidation state of the hemoprotein.

* Myoglobin is the "hemoglobin" found in muscle tissue. It differs from erythrocyte hemoglobin, which has 4 hemes/molecule and a molecular weight of about 68,000, in having 1 heme/molecule and a molecular weight of about 17,000. It resembles erythrocyte hemoglobin in all its chemical reactions and has the advantage that complicated effects, known as "heme-heme interaction," whereby the hemes in hemoglobin have different individual affinities for the ligand, of necessity are absent.

The accumulation of new experimental data during the past five years has now enabled a check to be made on the previous conclusions. The generalizations regarding the standard free energy changes and the manner in which the values of ΔH° greatly favor the hemoprotein reactions are further substantiated by the new data. However, a redetermination of the heat of oxygenation of ferromyoglobin makes a vast difference in the entropy values.[23] The new value, -9.7 kcal./mole in place of the old -16.4 kcal./mole, gives $\Delta S^\circ = -6$ eu instead of -30 eu. The quantity $(\bar{S}^\circ_{ML} - \bar{S}_M{}^\circ)$ is thus $+20$ eu, quite in accord with the values for the bonding of neutral ligands in simpler complexes. Thus the conclusion that an abnormal entropy change characterized this type of reaction no longer stands. It is to be hoped that values for the combination of isocyanides and independent values for the combination of CO and NO (rather than values obtained from the gas-exchange reactions O_2/CO, CO/NO) will soon be forthcoming to broaden the basis of comparison. It may be noted

TABLE 1

STANDARD FREE ENERGY CHANGES FOR Fe^{III} COMPLEX FORMATION AT 25° C.

In Kilocalories per Mole

Ligand	Fe^{+++}_{aq}	Mb	Hb	*Chiron.* Hb
OH^-	-16.1	-6.85	-7.1	-8.0
F^-	-6.9	-2.0	-2.4	-2.6
N_3	-5.7	—	-7.5	-6.6
CN^-	-9.6*	-11.5	—	—
CNO^-	—	—	—	-3.9
CNS^-	-4.2	—	-3.0	-4.6
$CNSe^-$	—	—	-2.9	-4.3
NO_2^-	—	—	-3.3	—
$H\cdot COO^-$	—	—	-2.0	—
$C_6H_5O^-$	—	-3.8	—	—

* See note to TABLE 5 of P. George.[20]

that another simple reaction has been studied,[24] which is analogous to these hemoprotein reactions,

$$(Co(NH_3)_5H_2O)^{++} + NO \rightleftharpoons (Co(NH_3)_5NO)^{++} + H_2O. \qquad (3)$$

The thermodynamic data are $\Delta F^\circ = -5.5$ kcal./mole, $\Delta H^\circ = -8.8$ kcal./mole, and $\Delta S^\circ = -11$ eu. The quantity $(\bar{S}^\circ_{ML} - \bar{S}_M{}^\circ)$ has the value $+18$ eu, somewhat less than 0.9 $\bar{S}_L{}^\circ$, 26 eu, but it is in keeping with the low value of $+10$ eu obtained for the $FeNO^{++}$ complex.

While the conclusion regarding the entropy change for the formation of complexes by the Fe^{II} oxidation state of myoglobin has needed revision, the new data for the complexes of the Fe^{III} oxidation state bear out the previous conclusion that the entropy change is far less favorable than it is in the Fe^{+++} aquo ion reactions. The thermodynamic data are brought together in TABLES 1 to 4 for comparison[20,25-27] (also, P. George, R. L. J. Lyster, and J. G. Beetlestone; unpublished results). Most of the new values are for reactions of erythrocyte and *Chironomus* hemoglobin. The old values for the azide complex have been recalculated by using the new value determined recently for the partial molal entropy of the azide ion, that is, 22.2 eu.[28]

TABLE 2
STANDARD ENTHALPY CHANGES FOR Fe^{III} COMPLEX FORMATION AT 25° C.
In Kilocalories per Mole

Ligand	Fe^{+++}_{aq}	Mb	Hb	*Chiron.* Hb
OH^-	−1.2	−7.65	−9.5	−9.6
F^-	7.5	−1.5	−2.5	−0.7
N_3^-	−4.3	—	−12.9	−10.2
CN^-	−4.8*	−18.6	—	—
CNO^-	—	—	—	−10.6
CNS^-	−1.5	—	−8.8	−11.7
$CNSe^-$	—	—	−5.6	−11.7
NO_2^-	—	—	−10	—
$HCOO^-$	—	—	−6.3	—
$C_6H_5O^-$	—	−2.1	—	—

* See note to TABLE 5 of P. George.[20]

TABLE 3
STANDARD ENTROPY CHANGES FOR Fe^{III} COMPLEX FORMATION AT 25° C.
In Entropy Units

Ligand	Fe^{+++}_{aq}	Mb	Hb	*Chiron.* Hb
OH^-	50	−2.6	−7.9	−5.0
F^-	49	+1.8	−0.6	+6.4
N_3^-	5	—	−18.4	−12.2
CN^-	16*	−24	—	—
CNO^-	—	—	—	−22.6
CNS^-	9	—	−19.7	−23.9
$CNSe^-$	—	—	−9	−24.7
NO_2^-	—	—	−22	—
$HCOO^-$	—	—	−14.6	—
$C_6H_5O^-$	—	+5.7	—	—

* See note to TABLE 5 of P. George.[20]

TABLE 4
$(\bar{S}^\circ_{ML} - \bar{S}^\circ_M)$ FOR Fe^{III} COMPLEX FORMATION AT 25° C.
In Entropy Units

Ligand	$\bar{S}^\circ_L$	Fe^{+++}_{aq}	Mb	Hb	*Chiron.* Hb
OH^-	−2.5	47.5	−5.1	−10.4	−7.5
F^-	−2.3	46.7	−0.5	−2.9	4.1
N_3^-	22.2	27.2	—	−3.8	−10.0
CN^-	28.2	42.3*	4.2	—	—
CNO^-	31.1	—	—	—	8.5
CNS^-	33.8	42.5	—	14.1	9.9
NO_2^-	29.9	—	—	8	—
$HCOO^-$	21.9	—	—	7.3	—
$C_6H_5O^-$	17.6	—	23.3	—	—

* See note to TABLE 5 of P. George.[20]

Summarizing, the quantitative data for the hemoprotein reactions show very clearly that one important effect that the chelate structure has on the reactivity of the iron is to make ΔH° for the bonding of ligands in the sixth coordination position especially favorable in relation to the corresponding aquo ion reactions. In addition, for the combination of anionic ligands with the Fe^{III} oxidation state, the entropy change is far less favorable, a fact that may be an indication that charge cancellation with its attendant desolvation effects does not play such an important role in the hemoprotein reactions as it does in those of the Fe^{+++} aquo ion.

Ligand Replacement Reactions of Vitamin B_{12} and Its Derivatives

Simple formation of complexes. As in the case of the hemoproteins, the great stability of the bonding within the heterocyclic ring system restricts the ligand replacement reactions of these cobaltic compounds to the fifth and sixth coordination positions. With aquocobalamin and factor B, ready replacement of the coordinated water molecules by many different ligands would be expected, according to the generalized reactions.

Aquocobalamin:

$$\begin{array}{c} H_2O \\ | \\ -Co^{III}- \\ | \\ benz \end{array} + L \rightleftharpoons \begin{array}{c} L \\ | \\ -Co^{III}- \\ | \\ benz \end{array} + H_2O \qquad (4)$$

Factor B:

$$NC{-}Co^{III}{-}OH_2 + L \rightleftharpoons NC{-}Co^{III}{-}L + H_2O \qquad (5)$$

Diaquo factor:

$$H_2O{-}Co^{III}{-}H_2O + L \rightleftharpoons H_2O{-}Co^{III}{-}L + H_2O \qquad (6)$$

$$H_2O{-}Co^{III}{-}L + L \rightleftharpoons L{-}Co^{III}{-}L + H_2O \qquad (7)$$

In the case of aquocobalamin several reactions of the type in formula 4 have been reported with, for example, CN^-, which regenerates cyanocobalamin, NO_2^-, SCN^-, and the nitrogenous bases NH_3 and histidine.[5,29-33] On treatment with CN^- the diaquo factor yields first factor B and then dicyano factor, according to reactions 6 and 7 and, with excess NO_2^-, it gives a derivative that is probably the dinitrito factor.[6] Ionization of the coordinated water molecule in aquocobalamin and one such molecule in the diaquo factor also have been observed,[6,29]

$$\left[\begin{array}{c} H_2O \\ | \\ -Co^{III}- \\ | \\ benz \end{array}\right]^+ \rightleftharpoons \begin{array}{c} OH \\ | \\ -Co^{III}- \\ | \\ benz \end{array} + H^+, \qquad pK \approx 6.9 \qquad (8)$$

$$[H_2O{-}Co^{III}{-}OH_2]^{++} \rightleftharpoons [H_2O{-}Co^{III}{-}OH]^+ + H^+, \qquad pK \approx 6.8 \qquad (9)$$

These reactions can be regarded equally as replacement of H_2O by the ligand OH^-.

To extend these observations, a series of simple experiments was carried out

by adding various ligands to dilute buffered solutions of factor B, and complex formation was characterized by following the changes in absorption spectrum from 300 to 600 mμ with a Beckman DU spectrophotometer. Like aquocobalamin, factor B reacts with neutral ligands such as nitrogenous bases and with anionic ligands such as OH^-, SCN^-, NO_2^-, CN^-. The main absorption band maxima for a number of these complexes are given in TABLE 5, and the spectra of the parent factor B and its NH_3 and CN^- complexes are illustrated in FIGURE 4. It is an interesting feature that all the complexes of factor B characterized thus far have spectra that correspond broadly to these three types. As may be seen from the table, the nitrogenous bases and the majority of the anions, despite their very different chemical natures, give complexes with the band in the ultraviolet at about 360 to 363 mμ, and bands in the visible at

TABLE 5

THE MAIN ABSORPTION BAND MAXIMA FOR FACTOR B, NC—Co^{III}—H_2O, AND FOR DERIVATIVES OF THE STRUCTURAL TYPE NC—Co^{III}—*L*, FROM 300 TO 600 Mμ

Ligand	Band in UV (mμ)	Bands in visible (mμ)	
Neutral groups			
H_2O (factor B itself)	355	498	531
NH_3	362	520	555
Imidazole	362	520	550
Histidine	361	520	552
Pyridine	363	515	550
CH_3NC	368	540	580
Anionic groups			
OH^-	362	520	553
CNS^-	361	520	550
NO_2^-	Ligand absorbs	515	550
N_3^-	363	522	552
$Fe(CN)_6^{---}$	361	525	555
$Fe(CN)_6^{---}$	Ligand absorbs	518	550
$Co(CN)_6^{---}$	360	520	550
CN^-	368	541	581

about 515 to 525 and 550 to 555 mμ. On the other hand, the bands for factor B are at 355, 498, and 531 mμ and for the CH_3NC and CN^- complexes, at 368, 540, and 580 mμ, although these are neutral and anionic ligands, respectively. The classification into these spectral types will be taken up again in a discussion of the reactions of factor B and vitamin B_{12} in acidic solution.

Several of the complexes listed in TABLE 5 call for special comment. First, a preliminary spectrophotometric determination of the *p*K for the ionization of the coordinated water molecule in factor B, that is,

$$[NC—Co^{III}—OH_2]^+ \rightleftharpoons NC—Co^{III}—OH + H^+ \qquad (10)$$

based on optical density measurements in buffers of different hydrogen-ion concentrations, places it at about 11 at 25° C. This higher value is in keeping with the lower value reported for the diaquo factor,[6] since the charge change in the ionization reaction is 1+ to zero instead of 2+ to 1+.

Second, the bonding of CH_3NC was investigated for the following reason. Vitamin B_{12} has been implicated in methyl group transfer reactions in biological systems,[34] and methylation of the N atom of the cyanide group would provide a very simple mechanism for this process (L. E. Orgel has independently proposed the same mechanism; personal communication, September 1959). A reaction of this kind is well known, for example, in the methylation of the ferrocyanide ion to give the hexamethylisocyanide ferrous ion,[35] that is,

$$Fe(CN)_6^{----} + 3Me_2SO_4 \rightleftharpoons Fe(CNMe)_6^{++} + 3SO_4^{--} \qquad (11)$$

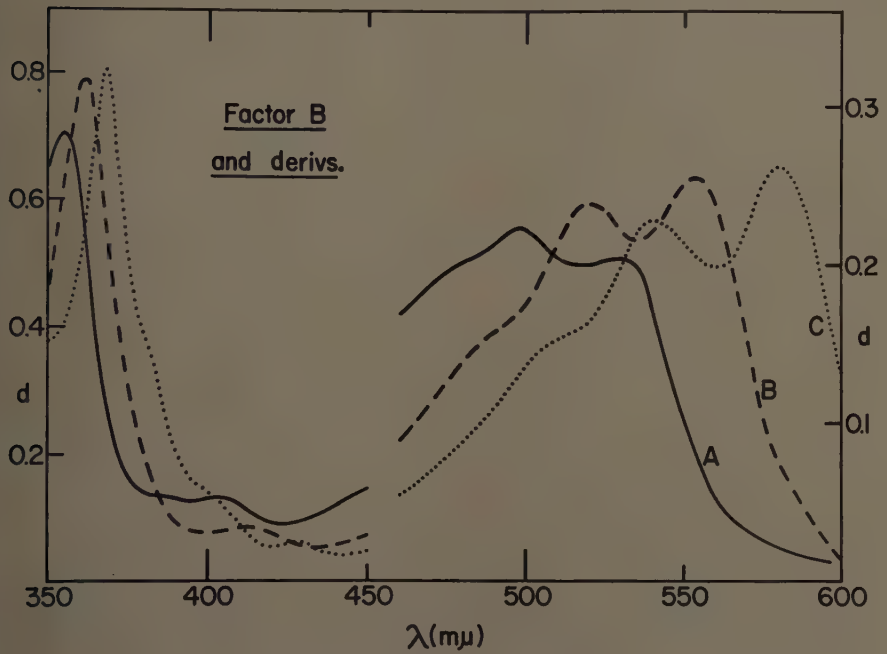
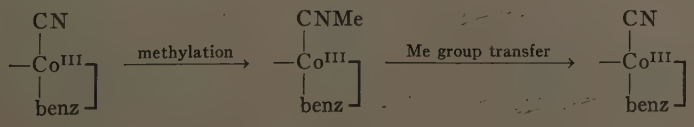


FIGURE 4. Absorption spectra of Factor B, its ammonia and its cyanide complexes: curve *A*, *B*, and *C*, respectively.

For this mechanism to be feasible in a biochemical system, the Co^{III}—CNMe complex must be reasonably stable, for otherwise it could not act as a transfer agent. An approximate value of 4×10^2 M^{-1} was obtained for the equilibrium constant for the formation of the factor B methylisocyanide complex by spectrophotometric titration, and this strongly suggests that aquocobalamin also would form a quite stable complex. Hence, if methyl group transfer is finally substantiated as a biochemical role of the vitamin, the above mechanism, which may be represented as



(12)

merits consideration. Although there is no obvious precedent with a simple inorganic complex, other one-carbon fragments than the methyl group also might be transferable in a similar manner.

Other complexes deserving of special mention are those formed with the ferrocyanide ion, and the related ferri- and cobalticyanide ions. No corresponding complexes thus far have been observed with hemin or the ferrihemoproteins. The possibility that complex formation is due to a cyanide group released from the hexacyanide ions, a dissociation that occurs readily in aqueous solution,[36,37] especially with the ferrocyanide ion,[38] can be ruled out on spectroscopic grounds because the cyanide complex of factor B has band maxima at very different wave lengths (TABLE 5). It appears that in these hexacyanide complexes the bonding involves coordination of one of the peripheral nitrogen atoms of a cyanide group, that is,

$$[NC—Co^{III}—OH_2]^+ + Fe(CN)_6^{----} \rightleftharpoons [NC—Co^{III}—NCFe(CN)_5]^{---} + H_2O. \quad (13)$$

The observation that the absorption bands of the hexacyanide complexes are at approximately the same wave lengths as are those for the complexes with nitrogenous bases is in keeping with such a formulation. Bonding of the cyanide ion between two metal ions in this way is known to be present in Prussian blue and related pigments,[39] but these factor B derivatives are apparently the first examples of such bonding in simple binuclear complexes.

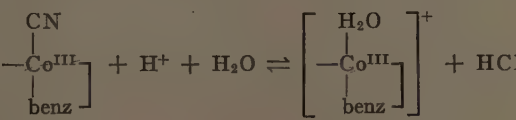
The following ligands were also tested, but no spectroscopic evidence was obtained to suggest that complex formation occurs: F^-, PO_4^{---}, CO, urea, $\alpha\alpha'$-dipyridyl, pyridine-2-aldoxime, phenol, and acetonitrile. In the case of $\alpha\alpha'$-dipyridyl and pyridine-2-aldoxime, steric hindrance probably prevents the complex from forming, since pyridine itself reacts quite readily. But this does not explain why acetonitrile does not react while the hexacyanide ions do. In these cases the high negative charge may be an important factor favoring complex formation through simple coulombic attraction and through a lowering of the ionization potential of a nitrogen lone-pair electron, which would facilitate electron donation.

A complicated reaction was observed when a solution containing the hydrosulphide ion SH^- was added to factor B. Initially, the color changes from a reddish orange to a violet, suggestive of the formation of the NC—Co^{III}—SH complex. However, this change is transient, and the solution rapidly reverts to its original color. A likely explanation is oxidation of the hydrosulfide by dissolved oxygen; however, further experiments using an inert atmosphere to check this were not carried out.

Reactions of vitamin B_{12} and factor B in acidic solution. The rapid reactions of aquocobalamin, factor B, and the diaquo factor with many ligands may be attributed to replacement of the water molecule or molecules that complete the coordination shell. On the other hand, in vitamin B_{12} the fifth and sixth positions are occupied respectively by the benzimidazole group of the nucleotide and by the cyanide group. Its simple replacement reactions therefore are restricted to ligands that have a greater affinity for the Co^{III} than for one or the other of these groups. For example, CN^- or SCN^- will replace the ben-

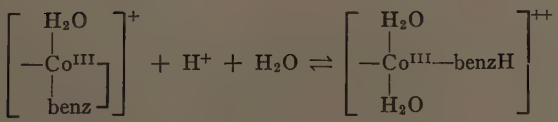
zimidazole, leaving the nucleotide bound to the ring system through only the phosphate ester and the amide linkages. Another type of replacement reaction can, however, be envisaged, in which the added reagent competes, not for the Co^{III} but for the benzimidazole or the cyanide, or both. In principle, a metal ion with a high enough affinity for these groups would be effective, and so would the proton, by combining to give the corresponding conjugate acid species. The gradual loss of HCN noted in acidified solutions of the vitamin,[29,40,41] and the acid hydrolysis of the nucleotide,[6] in which fission not only of the phosphate ester linkage but also of the Co^{III}-benzimidazole bond occurs, suggest that neither bond to the Co^{III} in vitamin B_{12} is so strong that it cannot be broken by competition with H^+ in solutions of moderate acidity. Since heating in 6 *N* HCl at 65° C. for 5 min. is required for complete nucleotide hydrolysis, a condition that also results in the hydrolysis of one of the amide groups, it seems unlikely that at room temperature and during the short time interval required for spectrophotometric measurements these irreversible reactions should occur to any appreciable extent. Hence, by varying the acidity it should be possible to study the reversible dissociation of both groups and to obtain the stability constants.

Depending on the relative affinities of the CN^- and benzimidazole groups for the Co^{III} and H^+, one of two reaction sequences would be favored as the acidity is increased:



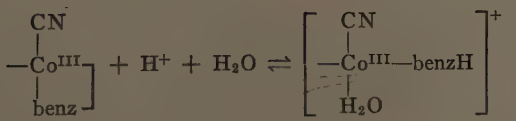
(14)

followed by



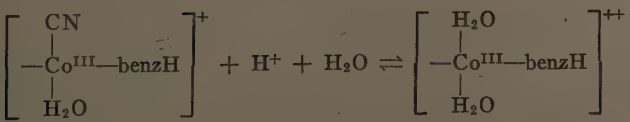
(15)

or



(16)

followed by



(17)

The intermediate species in the first sequence is aquocobalamin and in the second, a derivative analogous to factor B but containing the nucleotide with the benzimidazole in its conjugate acid form. Once it is split from the Co^{III}, the nucleotide is many atoms distant from the chromophore, and therefore it is reasonable to assume that, apart from its own absorption in the region of 260

to 290 mμ, it no longer will influence the main chromophore absorption at about 350 to 370 mμ and throughout the visible region. If the dissociation follows the second sequence of reactions, 16 and 17, the spectrum should change to the spectrum of factor B and then to that of diaquocobalamin, as acidity is increased. Furthermore, in very acidic solution, both vitamin B_{12} and factor B should show the same chromophore absorption, since complete dissociation results in identical coordination about the cobalt, with two water molecules bonded in the diaquo structure: $H_2O—Co^{III}—H_2O$.

A series of experiments was carried out at approximately 25° C. in which varying amounts of HCl were added to a solution of vitamin B_{12} in water (0.100 gm./l.), to give hydrogen-ion concentrations ranging from 10^{-2} to 2.0 *M*. The absorption spectra were recorded on a Cary spectrophotometer from 275 to 600 mμ; from the addition of the acid to the completion of the recording about 6 min. elapsed. Over this range of acidity a progressive change in spectra was noted, the band at 362 mμ moving finally to 356 mμ and the bands at 520 and 552 mμ moving to 498 and 530 mμ, respectively, but at intermediate

TABLE 6

ABSORPTION BAND MAXIMA OF VITAMIN B_{12} IN WATER AND IN ACIDIC SOLUTIONS

Water, *p*H 6.7							
λ (mμ)	279	306	323	362	—	520	552
ε (mmoles)	16.8	10.1	8.6	29.6	—	8.7	9.6
HCl, 0.15 *M*							
λ (mμ)	279	306	323	362	—	520	551
ε (mmoles)	17.2	10.0	8.8	29.6	—	8.6	9.2
HCl, 2.0 *M*							
λ (mμ)	277	—	322	356	405	498	530
ε (mmoles)	20.8	—	12.2	29.3	5.4	8.9	8.6

acidities each spectrum was found to remain unchanged with time. This indicates that the dissociation equilibriums are established very rapidly, and the data therefore can be used to calculate stability constants.

With increasing acidity the change in spectrum was found to follow a certain pattern. Up to about 0.15 *M* HCl the wave lengths for maximal absorption remain virtually unaltered, but small reproducible changes in extinction coefficients occur, as shown in TABLE 6. The peaks at 279 and 323 mμ increase in intensity, the 552-mμ peak decreases, while those at 306 and 362 mμ are almost the same. Furthermore, there is no change in the slight shoulder that appears on the longer wave-length side of the 279-mμ benzimidazole peak, evidence that the Co^{III}-benzimidazole bond is still intact.[42]

As the acidity is increased above 0.15 *M* HCl, both the position and intensities of the absorption bands alter, and at 2.0 *M* HCl the change is complete (TABLE 6). A substantial increase in the intensity of the benzimidazole band occurs and, in addition, the absorption previously present as a shoulder is now resolved into a small discrete band maximum giving the appearance of a notch characteristic of uncoordinated benzimidazole.[42] In the 2.0 *M* HCl solution the main chromophore absorption at about 360 mμ and throughout the visible is, as anticipated, very similar indeed to that of factor B in strongly acidic

solution. The absorption data obtained in a parallel experiment with factor B and a comparison of intensities are given in TABLE 7. The comparison is made by taking ratios of intensities, since exact molar extinction coefficients of factor B were not available. The discrepancy of 4 mμ in the position of the long wave-length band is not significant, because it has a very broad, flat maximum.

These successive changes in the vitamin B_{12} spectrum are compatible with only the first reaction sequence set out above, reactions 14 and 15, where removal of the CN group precedes fission of the Co^{III} benzimidazole bond, giving aquocobalamin as the intermediate. The other reaction sequence, 16 and 17, where the factor B type derivative is the intermediate, would be characterized by changes in both intensity and band maxima from the start, with a simultaneous appearance of the notch on the benzimidazole peak. However, the changes in spectrum all occur within a *p*H range of about 2 units, which indicates that the two dissociation reactions overlap to a large extent. In trial calculations based on the change of optical density with *p*H at 500 and 552 mμ

TABLE 7

RATIOS OF MAIN BAND INTENSITIES OF VITAMIN B_{12} AND FACTOR B IN STRONG ACID, WITH REFERENCE TO THE LONG WAVE-LENGTH BAND

Vitamin B_{12} in 2.0 *M* HCl			
λ (mμ)	356	498	530
ε (mmoles)	29.3	8.9	8.6
Ratios	3.41	1.04	1.00
Factor B in 1.5 *M* HCl			
λ (mμ)	354	497	527
$E^{1\%}_{1cm.}$	166	53	49
Ratios	3.39	1.08	1.00

it was found that two separate dissociation constants could not be obtained readily, but the data were reasonably consistent with a value of 10^{-3} M^{-1} for the over-all two-step dissociation:

$$K_{diss} = \frac{\left[\begin{array}{c} H_2O \\ | \\ -Co^{III}-benzH \\ | \\ H_2O \end{array}\right] [HCN]}{\left[\begin{array}{c} CN \\ | \\ -Co^{III}- \\ | \\ benz \end{array}\right] [H]^2} \qquad (18)$$

Now, if $K_{CN(1)}$ and K_{benz} denote the stability constants for the bonding of the first cyanide and the benzimidazole to the cobalt, a comparison of the component equilibrium expressions shows that the product of $K_{CN(1)} \cdot K_{benz}$ can be evaluated from the equation

$$K_{CN(1)} \cdot K_{benz} = \frac{1}{K_{diss} \cdot K_{HCN} \cdot K_{benzH}} \qquad (19)$$

where K_{HCN} and K_{benzH} are the ionization constants for HCN and the benzimidazolium group, respectively. Using the values 4×10^{-10} M and 1.1×10^{-6} M for these constants,[43,44] the value of 10^{18} M^{-1} is obtained for the product $K_{CN(1)} \cdot K_{benz}$ to the nearest power of 10. Approximate values for the two individual stability constants can be obtained, however, from the following considerations. It was found that with the vitamin concentration employed the cyanide dissociation equilibrium was measurable at $[H^+] \approx 10^{-1}$ M and was virtually complete at $[H^+] \geq 1.0$ M. This gives a value of $K_{CN(1)} \approx 10^{12}$ M^{-1}; hence $K_{benz} \approx 10^6$. If $K_{CN(1)}$ were greater than 10^{13} M^{-1}, the benzimidazole dissociation would have occurred at a lower hydrogen-ion concentration than did the cyanide dissociation and, on the other hand, if $K_{CN(1)}$ were less than 10^{11} M^{-1}, then the cyanide dissociation equilibrium would have been measurable at hydrogen-ion concentrations much less than 10^{-1} M.

In view of the overlap of the two dissociation steps, the spectrum at no stage corresponds entirely to aquocobalamin. Nevertheless the trend in the optical densities for the solutions of acidities up to 0.15 *M* HCl indicates that aquocobalamin would have an absorption spectrum with the main bands at wavelengths almost identical with those of cyanocobalamin and with only slightly different extinction coefficients. In other words, the replacement of H_2O by CN^- results in very little spectral change. A similar effect has been noted previously in the reaction of one of the two water molecules in the diaquo factor.[6] However, the replacement of a water molecule by the benzimidazole or the bonding of a second cyanide group, results in substantial changes in both band position and intensity. These effects are illustrated schematically in FIGURE 5, where the replacement reactions indicated by the vertical arrows are those accompanied by marked changes in band position and intensity, and those indicated by the horizontal arrows by slight changes in intensity alone. Substitution of H_2O by CN^- normally results in a spectral change comparable to that produced by the coordination of nitrogenous bases; hence this anomalous behavior of aquocobalamin and the diaquo factor presents a problem to the theoretical chemist. A closely related problem is the classification of factor B and its derivatives into the three main spectral types commented on (TABLE 5). Undoubtedly these are all low-spin complexes, so no correlation is possible as in the case of hemoprotein derivatives where, for the same oxidation state, the high-spin complexes have one type of spectrum and the low-spin complexes have another.[45,46]

It may be noted that spectra for aquocobalamin reported in the literature[30,31] have the main chromophore bands at 351 to 353 mμ and at 520 to 530 mμ, which do not correspond to the values inferred from the above dissociation experiments, namely, at about 360, 520, and 550 mμ. These data refer to samples prepared from cyanocobalamin by photolysis or catalytic hydrogenation to remove the cyanide, subjected to oxidation (in the latter method) and purification steps and then isolated in solid form. An exploratory study of one sample of this kind gave the following indications that derivatives other than aquocobalamin can be present. First, in fresh neutral solution the main ultraviolet band was at 355 mμ, but on standing 24 hours it shifted to 351 mμ, a change that occurred far more rapidly in acidic solution. Second, the addition of a twofold excess of cyanide to a fresh neutral solution did not regenerate

cyanocobalamin. Third, the addition of excess cyanide did not result in the immediate formation of the dicyano derivative and, even on standing, only about 70 per cent was formed. Under similar conditions vitamin B_{12} reacts immediately, as aquocobalamin should. On treating the solution obtained in the second experiment with excess imidazole, a change in the spectrum consistent with the binding of more of the cobalt in an "NC—Co^{III}—nitrogenous base" linkage was obtained. These observations suggest that in this material

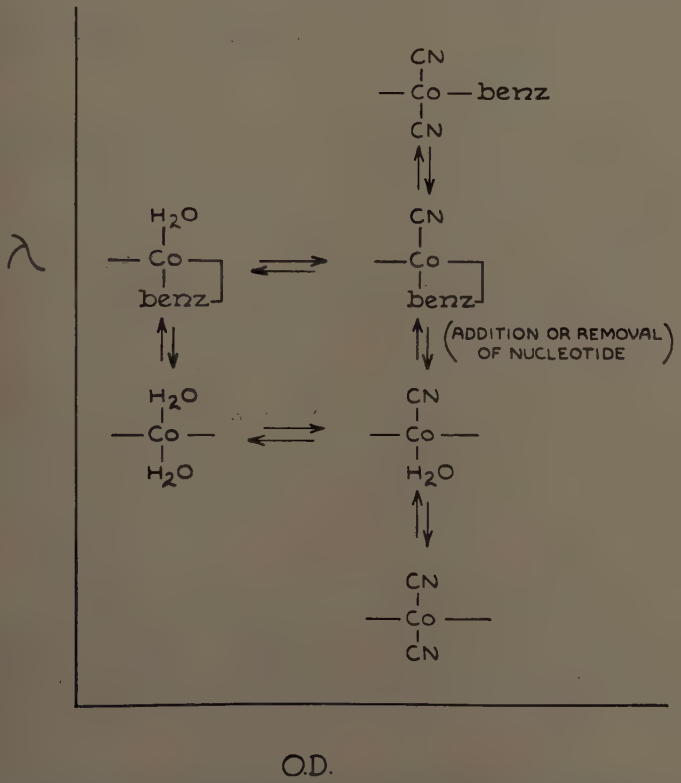


FIGURE 5. This diagram shows that the replacement reactions indicated by the vertical arrows are accompanied by marked changes in both band position and intensity, whereas those indicated by the horizontal arrows are accompanied by only slight changes in intensity.

the nucleotide may have been partly or entirely split from the cobalt, and some other group or groups firmly bound in the fifth and sixth coordination positions, which dissociate relatively slowly.

Reaction of vitamin B_{12} and factor B with cyanide. Excess cyanide readily replaces the water molecule in factor B and the benzimidazole group in vitamin B_{12}. In the resulting dicyano complexes the coordination about the cobalt is identical, and both derivatives have the same absorption characteristics for the main chromophore bands at 368, 541, and 581 mμ to within experimental error (TABLE 8). The release of the benzimidazole group in the case of cyanocobala-

min leads to the appearance of a small discrete band at 289 mμ, and the notch referred to previously.[42]

Spectrophotometric titrations of cyanocobalamin and factor B with cyanide at 500 and 580 mμ, respectively, were carried out at 25° C. From the optical density changes, the following values (rounded off to the nearest power of 10) were obtained for the equilibrium constants of the two reactions:

$$[NC—Co^{III}—OH_2]^+ + CN^- \rightleftharpoons NC—Co^{III}—CN + H_2O, \qquad K'_{CN(2)} = 10^8\ M^{-1}$$

$$\begin{matrix} CN \\ | \\ —Co^{III}— \\ | \\ benz \end{matrix} + CN^- \rightleftharpoons \left[\begin{matrix} CN \\ | \\ —Co^{III}—benz \\ | \\ CN \end{matrix}\right]^-, \qquad K_{CN(2)/benz} = 10^4\ M^{-1}$$

The suffix CN(2) indicates that the constant is for the bonding of the second cyanide group, and the suffix CN(2)/benz indicates that cyanide replaces benzimidazole in this reaction.

TABLE 8

ABSORPTION DATA FOR THE DICYANO COMPLEXES FROM VITAMIN B_{12} AND FACTOR B AND THE RATIOS OF EXTINCTION COEFFICIENTS, WITH REFERENCE TO THE LONG WAVE-LENGTH BAND (581 MILLIMICRONS)

Vitamin B_{12}								
λ (mμ)	278	289	308	313	368	417	541	581
ε (mmoles)	16.3	12.3	10.9	10.8	34.4	2.6	9.8	11.1
Ratio	1.47	1.11	0.98	0.97	3.10	0.23	0.88	1.00
Factor B								
λ (mμ)	277	—	308	313	368	418	541	581
$E^{1\%}_{1cm.}$	75	—	68	67	191	16	55	62
Ratio	1.21	—	1.10	1.08	3.08	0.26	0.89	1.00

These values enable several other important stability constants to be estimated. From the dissociation equilibriums, $K_{CN(1)} \cdot K_{benz}$, the product of the stability constants for the bonding of the first CN^- and the benzimidazole was found to be $10^{18}\ M^{-1}$. Since the equilibrium constant for the replacement of benzimidazole by CN^- is $10^4\ M^{-1}$, the product of the stability constants for the bonding of both CN^- groups, $K_{CN(1)}$ and $K_{CN(2)}$, is $10^{22}\ M^{-2}$. This value of $10^{22}\ M^{-2}$ also may be adopted for the corresponding product in the case of factor B because, once the benzimidazole-Co^{III} bond is broken, the nucleotide remains attached to the molecule only via the long side chain and can be assumed to influence no longer the affinity of the Co^{III} for ligands. For factor B, therefore, if $K'_{CN(1)} \cdot K'_{CN(2)} = 10^{22}\ M^{-2}$, and $K'_{CN(2)} = 10^8\ M^{-1}$, it follows that $K'_{CN(1)} = 10^{14}\ M^{-1}$. These stability-constant relationships are brought together for comparison in FIGURE 6. An interesting feature may be noted in the difference between $K_{CN(1)}$ and $K'_{CN(1)}$, namely, 10^{12} and $10^{14}\ M^{-1}$, respectively. The former is for the bonding of the cyanide to aquocobalamin; the latter, for the bonding of the first cyanide to the diaquo factor. This difference of 10^2 thus represents the change in the affinity of the Co^{III} for CN^- brought about by the substitution of H_2O by benzimidazole, since the other four coordinated groups remain the same.

These cyanide stability constants are almost of the same order of magnitude as the average value in the case of the cobalticyanide ion $Co(CN)_6^{---}$, for which the product of the six constants[47] is given as 10^{64} M^{-6}. Undoubtedly, the value for the first constant is appreciably greater than 10^{11} M^{-1}, and the value for the last, much smaller, because of charge effects. The stability constant for the bonding of the benzimidazole, 10^6, is also very similar in magnitude to the average value for the $Co(NH_3)_6^{+++}$ complex, for which the over-all constant[48] is given as 10^{34} M^{-6}. However, in this case there is the important difference that the dissociation of the Co—benzimidazole bond is an intra-

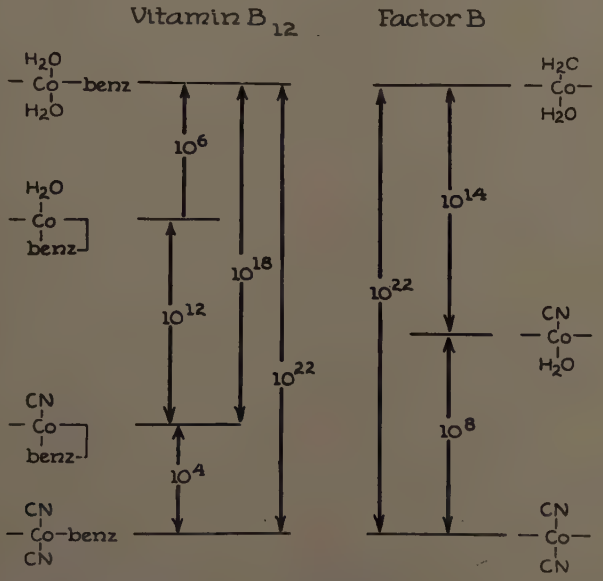


FIGURE 6. This diagram summarizes the relationships between the various stability constants, given to the nearest power of 10, for the bonding of cobalt to the benzimidazole and cyanide groups in vitamin B_{12}, and to the cyanide groups in dicyanocobalamin, factor B, and the dicyano factor.

molecular reaction. These values do not suggest that coordination of the cobalt in the special heterocyclic ring of vitamin B_{12} has resulted in an enhancement of its affinity. On the other hand, the cyanide stability constant $K_{CN(1)}$ of 10^{12} M^{-1} makes very clear why aquocobalamin should be so very efficient in combining with cyanide and, conversely, why cyanocobalamin is not toxic through the release of cyanide.[49]

Stabilization of Oxidation States by the Chelation Systems

Although stability constants for the bonding of the individual metal ions in the hemoproteins and cobalamins are lacking almost completely and although those for the bonding within the heterocyclic rings will prove exceptionally difficult to obtain,[50] since a knowledge of the *p*K values for the ionization of the NH groups in true aqueous solution will be required, a certain amount of

information on the relative values of the over-all stability constants is provided by the ordinary oxidation-reduction reactions.

The value of E_0 for the Fe^{+++}/Fe^{++} aquo ion couple is +0.77 volts (v),[51] compared to E_0 values for the Fe^{III}/Fe^{II} couples of myoglobin, hemoglobin, and cytochrome *c* of +0.12, +0.16, and +0.2 v, respectively, at 25° C. in the *p*H range 6 to 7.[52,53] This difference of about 0.6 v implies that the over-all stability constant for the bonding of the Fe^{+++} ion in the ferrihemoprotein is some 10^{10} times greater than the corresponding constant for the bonding of the Fe^{++} ion in the ferrohemoprotein.

Another more striking comparison is made between the value of 0.2 v for cytochrome *c* in relation to that of 1.12 v for the $Fe(dipyridyl)_3^{+++}/Fe(dipyridyl)_3^{++}$ complex ion couple.[54] Both Fe^{II} and Fe^{III} forms of each couple are low-spin octahedral complexes in which six N atoms are bonded to the iron. Yet there is an even greater difference between the E_0 values than in the previous comparison with the aquo ion and, in contrast to the hemoproteins, the Fe^{II} state is stabilized with respect to the Fe^{III} state in the trisdipyridyl complexes by a factor of about 10^6 in the stability constants.

In hemoglobin and myoglobin, at least the group attached in the sixth coordination position is different from that in cytochrome *c*; nevertheless, this does not result in any substantial change in the E_0' values. Furthermore, although all these hemoprotein values show a trend toward greater stabilization of the Fe^{II} state, when compared to the E_0' value of −0.1 to −0.2 v reported for the Fe^{III}/Fe^{II} iron porphyrin couple at *p*H 9.15,[55] this trend is relatively small in magnitude, when the over-all range of values including those for the aquo ions and trisdipyridyl complex ions is considered. This value for the iron porphyrin couple is of dubious thermodynamic significance, because both oxidized and reduced species are present as molecular aggregates in water[56,57] but it is somewhat unlikely that, if the value for monomeric species in aqueous solution were known, the sequence of E_0' values would be grossly altered. Uncertainty on this point does not affect the broad conclusion that, with the hemoproteins, the chelation already present in the porphyrin ring very largely determines the magnitude of the oxidation-reduction potential for the Fe^{III}/Fe^{II} couple and hence the reactivity of the Fe^{II} and Fe^{III} states in electron-transfer processes.

An important elaboration of these comparisons is the determination of ΔH° and ΔS° for the standard cell reactions, that is,

$$Fe^{III} + \tfrac{1}{2}\, H_2 \rightleftharpoons Fe^{II} + H^+.$$

Data for the aquo ion and trisdipyridyl complex ion couples already show that ΔH° and ΔS° make significantly different contributions in these cases.[54] The values of ΔS° indicate far less solvent interaction with the complex ions. Preliminary studies of hemoglobin and myoglobin[52] suggest that, in terms of the partial molal entropies, the Fe^{III} state is favored with respect to the Fe^{II}. This is rather a surprising result because, on the basis of the charge neutralization involved in going from Fe^{III} to Fe^{II}, the opposite effect would be expected.

No E_0' values for cyano- and aquocobalamin have been reported, and there is some evidence that on reduction to the Co^{II} state the compounds are rela-

tively unstable and the cobalt is rather readily removed from the heterocyclic ring. This in itself is a qualitative indication that, as with the hemoproteins, coordination of the +3 oxidation state is far more favored than the +2 state. Further support is afforded by the observation that these Co^{III} compounds are poor oxidizing agents in aqueous solution, quite unlike the Co^{+++} aquo ion, for which $E_0 = 1.84$ v.[58] In all probability, the difference in E_0 would amount to about 1.0 to 1.5 v, equivalent to a difference of 10^{17} to 10^{25} times in the overall stability constant favoring the formation of the Co^{III} compound.

A few experiments on the reduction of factor B were carried out to see whether it behaves in the same way as cyanocobalamin. On treatment with $Na_2S_2O_4$, a marked change in spectrum was observed. In place of the bands in the visible there is very low absorption, increasing steadily from 600 mμ to a broad flat maximum in the region of 430 mμ, with another band at about 340 mμ. Addition of H_2O_2 regenerated 80 per cent of factor B. Similar results were given by β-mercaptoethanol, but with KBH_4 considerable destruction was noted.

With the assumption that the compound formed by $Na_2S_2O_4$ reduction is the Co^{II} analogue of factor B, reasonable in view of the rapid regeneration of factor B by H_2O_2 oxidation, certain features of its spectrum call for comment. While all the Co^{III} compounds have their strongest absorption bands in the region of 350 to 370 mμ, between 3 and 4 times more intense than their visible bands (*see* FIGURE 4), the Co^{II} derivative has a much lower absorption, no greater than that of the visible bands of the Co^{III} compounds. At the maxima, taking the intensity of the 581 mμ band of the dicyano factor as an arbitrary standard of unity, the bands of the Co^{II} derivative have relative intensities of 1.0 at 430 mμ and 1.15 at about 340 mμ.

These spectral data suggest the interesting possibility that the adenylcobamide coenzyme, which has been isolated recently,[59] is a cobaltous derivative. In this coenzyme form of pseudovitamin B_{12} the nucleotide contains adenine in place of 5:6 dimethylbenzimidazole and another adenine in place of the cyanide. Its spectrum (*see* FIGURE 4 in Barker *et al.*[59]) is very similar to that of the Co^{II} derivative described above. If the hypothesis proves correct, then on addition of excess cyanide, which gives a derivative with the typical spectrum of a dicyano—Co^{III} compound, oxidation also must occur. Magnetic susceptibility measurements would provide a means of testing this hypothesis, since a Co^{II} derivative would be paramagnetic, whereas the Co^{III} compounds are diamagnetic.

In concluding this survey of oxidation states, very brief mention must be made of the reactions of ferrihemoproteins with peroxides and other strong oxidizing agents, which yield compounds having the oxidation-reduction characteristics of Fe^{IV} and Fe^{V} states. With ferrimyoglobin and ferrihemoglobin a single derivative is formed, which is 1 equiv. above the Fe^{III} state.[60] It is a potent oxidizing agent, with $E_0' = 0.9$ v at *p*H 7.0 and 25° C.[61] There is substantial evidence that it has either the "ferryl ion" structure, that is, Prot—Fe—O, or a structure isomeric with this.[61] With ferriperoxidase and ferricatalase two such derivatives are formed, 1 and 2 equiv. above the ferric state, respectively,[62,63] the former having a spectrum different from the hemoglobin and myoglobin derivative. Ferriperoxidase reacts in this manner with many

strong oxidizing agents,[64] but only H_2O_2, alkyl hydroperoxides[65] and, to a lesser extent, HOCl,[66] have been found effective with ferricatalase.

The reason why ferriperoxidase and ferricatalase give these two derivatives, while ferrimyoglobin and ferrihemoglobin give only one, may be intimately connected with a difference in the structure of the Fe^{III} state, as revealed by the *p*H variation of the equilibrium constants for the bonding of ligands.[8] An important question is whether the site of oxidation is the iron atom or some other center in the molecule that bears a close structural relationship to it. Like the myoglobin and hemoglobin derivatives and unlike the Fe^{II} and Fe^{III} states, these higher oxidation states do not react readily with neutral or anionic ligands; thus it may be inferred that a group newly formed and essential to their reactivity is firmly bonded in the sixth coordination position. This inference is borne out by the observation that ferricytochrome *c* does not react to give any higher oxidation state.[67] Attention is drawn to recent articles in which various structural hypotheses are discussed.[8,20,68,69]

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USE OF METAL CHELATES FOR PLANT TISSUE CULTURES*

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That plants require minute amounts of metal ions has been known for more than 75 years. Indeed, the practice of driving nails into chlorotic trees undoubtedly goes back more than 100 years. Iron was, of course, the first of the so-called micronutrient elements to be recognized, and the need of manganese, zinc, copper, and boron for plant growth was demonstrated by the start of the 1920's. More recently, cobalt, molybdenum, nickel, and gallium have been shown to be vital to plant growth. As our techniques for purifying salts used in the preparation of nutrient solutions improve, it is possible that even more elements will be shown to be required. Thirty-five elements have been found in plant tissues, and proof of essentiality has been provided for less than 20.

These trace levels of metal ions in nutrient solutions are provided deliberately by the addition of Bertholet's *oligo-dynamique* and Hoagland's "A-Z" solutions, or indirectly through the impurities present even in the best grades of reagent chemicals. The latter fact alone gives some idea of how extremely small are the requirements for many of these ions.

However, even these low levels of metal ions frequently are not attained, in spite of deliberate additions. In a nutrient solution containing initially 20 or more ions, complex interactions may occur in which natural chelates bind or actually precipitate a metal ion (Hutner *et al.*, 1950). The coprecipitation of manganese with phosphate salts is well known. Metal ions may be bound on surfaces—the interaction of calcium and zinc is an example. Finally, by far the most troublesome consequence of the use of complex nutrient solutions is the precipitation of metal ions. Inorganic salts of manganese, zinc, nickel, molybdenum and, particularly, iron are generally soluble at *p*H values below 5.5, a *p*H chosen for many solutions. Consider, however, the shifts in *p*H of a nutrient solution that are attendant upon differential ion uptake. When ni-nitrate and phosphate ions are taken up by cells at more rapid rates than are calcium or magnesium, the *p*H of the medium frequently rises several units. When the *p*H rises above 6, iron becomes unavailable and, when *p*H 7 is reached, copper, zinc, and cobalt become limiting. The products of metabolism may alter further the *p*H, and the biosynthesis of such natural chelating agents as citric acid may complicate the picture beyond analysis.

The Concept of Metal-Buffering

The investigator may use several methods of providing adequate levels of metal ions. Where sterility is not a problem, as in hydroponic gardens, sand cultures, frequent additions of metal ions, and periodic adjustment of the *p*H may suffice. Here, however, one cannot possibly talk about constant conditions. Such additions are very difficult to control when sterility is a factor. A second method is by frequent transfers to fresh medium. By far the simplest

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and most direct way of solving the problem is provided by deliberate chelation of the metals. In so far as we have been able to determine, the first precise statements on this subject were by Schwarzenbach (1949) and by Schatz and Hutner (1949*b*), who suggested that metal-buffering through chelate formation would result in the maintenance of nearly constant concentrations of metal ions over a long period of time.

Their analogy of metal-buffering to *p*H-buffering is quite apt. Like the man who was astounded to learn that he had been speaking prose all his life, botanists had used chelates such as iron tartrate or iron citrate in nutrient solutions since 1923, but were unable to explain their good results. They recognized the inherent theoretical difficulties in using organic molecules, which are readily metabolized, and except in mycological work such chelates were not used extensively.

Metal-Buffering for Microbiological Culture Media

The pioneer investigations into the use of chelating agents in biological media were those of Hutner and his collaborators. Schatz and Hutner (1949*a*) recognized that the constituents of media can form complexes with the metal ions needed and suggested that this fact be considered in formulating nutrient solutions. In an elegant paper, Hutner *et al.* (1950) critically examined the known approaches to the problem of the role of metals in the metabolism of microorganisms. In this and subsequent papers, the properties of ethylenediaminetetraacetic acid (EDTA) as a metal-chelating and solubilizing agent were brilliantly exploited. Provasoli and Pintner (1954) and Hutner and Provasoli (1951) developed the concepts and provided a rational approach to the use of EDTA. Hutner *et al.* (1957) demonstrated that the green flagellate *Ochromonas* had greatly increased metal ion requirements under conditions of temperature stress—a phenomenon that workers in other fields might profitably examine. They also developed the use of dry mixes of metals, which permits indefinite expansion of work on these problems. Provasoli *et al.* (1957) using EDTA as a solubilizing and metal-buffering agent, were able to avoid the use of soil extracts for the growth of marine algae. To date these findings have not been used extensively. Davis *et al.* (1953) found that the addition of EDTA to media used for *Chlorella* permitted a 68 per cent increase in growth. Walker (1953) reported that EDTA was a useful addition to *Chlorella* media, and both French (1953), Beresford (1953), and Waris (1953) reported that EDTA permitted better growth of green algae *in vitro*. Sweeney (1954) included EDTA in the medium developed for *Gymnodinium* and reported that reproducible results were obtained only in this medium. The media used for *Achyla* (Reischer, 1951; Barksdale, 1960) also contained EDTA as a solubilizing agent.

Metal Chelates in Nutrient Solutions

In view of the dramatic effects on chlorotic plants of the addition of chelated iron to alkaline soils (cf. Wallace, 1956), it is surprising that so little use has been made of these findings for growing plants under conditions of controlled nutrition. Jacobson (1951) observed that iron deficiences were common in

tomato and other plants grown in nutrient solutions. He found that a single addition of an Fe-EDTA chelate was sufficient for an entire experiment in spite of alkaline drifts in *p*H with time. This technique was a distinct advantage over the usual practice of adding iron at weekly or biweekly intervals. Perhaps some of the reluctance of investigators to use metal chelates was due to the rather unfavorable reports of Heck and Bailey (1950) and of Rasmussen (1956), to the effect that the addition of EDTA to nutrient solutions removed necessary ions from solution. Its use, however, for growing delicate orchids from seed has been reported (Menninger, 1954), and it is clear that EDTA cannot be indiscriminately used. Majumdar and Dunn (1956) found that copper toxicity in nutrient culture of corn was alleviated by chelated iron applied as a foliar spray or added to the substrate. Weinstein *et al.* (1954) found that Fe-EDTA moved as such through the root system and tops of sunflower plants and effectively supplied the necessary iron. More recently, Hillman (1959) used EDTA as a solubilizing agent in the complex medium required by *Lemna*.

Chelating Agents as Auxins

No discussion of EDTA in relation to plant growth would be complete without reference to its possible role in cell enlargement. Bennett-Clark (1956) suggested that indole-3-acetic acid may function in extension growth of plant cells by virtue of its capacity to chelate the calcium known to be involved in the plastic extension of cell walls. Heath and Clark (1956*a* and *b*) reported that chelating agents such as EDTA or 8-hydroxyquinoline were, in fact, auxins as operationally defined. They observed that EDTA, like indole-3-acetic acid, suppressed the elongation of wheat roots. Weinstein *et al.* (1956*a*) found that auxin or EDTA at comparable concentrations increased water uptake and elongation of etiolated lupin seedlings. When complexed with iron, however, the chelate caused a slight decrease in elongation and water uptake. These workers also reported (1956*b*) that flowering was accelerated by EDTA treatments. Burström and Tullin (1957) found, as reported by Heath and Clark (1956*a*), that EDTA suppressed root growth. This suppression, however, was due to a deficiency in cell multiplication, whereas auxin suppressed only cell elongation. Furthermore, the EDTA effect was relieved by calcium ions which do not reverse the auxin effect. Most recently, investigations by Thimann (1960) have indicated that the effects of EDTA and auxin are clearly different and that EDTA, although not an auxin, acts in other ways. As will be shown in this report, EDTA is effective in increasing the growth of plant tissue cultures that are prototrophic for auxin.

Metal Chelates for Plant Tissue Culture Media

Through the pioneer work of Gautheret (1959), White (1953), and others, many plant tissues of diverse morphogenic and etiological backgrounds have been established as sterile cultures on defined media. In the hands of many plant physiologists, these tissue cultures have provided a new tool for physiological investigations of growth, differentiation, and other processes.

Several formulas have been developed for plant tissue cultures, but examination of their composition indicates that there is relatively little difference

among them. All contain approximately the same inorganic ion composition, none is well buffered, and most have an initial *p*H of about 5.3. Claims of significantly increased growth of cultures on one or another of these media have not always been substantiated. One of the more vigorous tissue cultures, crown gall of *Parthenocissus tricuspidatus*, showed two- to threefold increases in fresh weight in a three-week period. As estimated from mitotic indices, cell generation time is measured in terms of hours or even days. When compared with the rates of division of cells in bud primordia, root tips, or other rapidly growing plant tissues, rates of cell division in tissue cultures are at a very low level.

All this led us to postulate that available media may be far from optimal. In view of the excellent and critical work on the composition of media by White, Gautheret, Hildebrandt and Riker, Heller, and others (cf. Gautheret, 1959), it seemed unlikely that extensive modification of the quantity or the composition of the major components of available formulas would lead to much greater growth. On both theoretical and empirical grounds, supplementation of the medium with characterized or uncharacterized growth factors (such as coconut milk or plant extracts) may be contra-indicated. Finally, a critical study by White (1953) indicated that some essential nutrient is depleted more rapidly in liquid than in solidified standard medium.

A discussion of these considerations with H. E. Street, University of Swansea, Wales, pointed up the possibility that the availability of "trace metals" might be one of the limiting factors in the cultures of plant tissues. Street *et al.* (1952) and Boll (1954) reported that "staled" medium in which tomato roots had ceased to grow could be rejuvenated by the addition of ferric ions coupled with adjustment of the *p*H from an alkaline to an acid value. They concluded that alkaline drift, caused by differential ion uptake, caused the precipitation of iron. Finally, they found that a preformed chelate of iron and EDTA added to the medium would prevent the staling even when the *p*H approached neutrality. Street (1957) later reported that this chelate was effective at a *p*H of 7.2.

Media used for plant tissue cultures have an initial *p*H of 5.0 to 5.5. As growth proceeds, the *p*H drifts toward neutrality (Hildebrandt *et al.*, 1945). Alterations in *p*H toward neutrality would, of course, result in the removal of the iron and other metal ions, which are absolutely required for growth (Hildebrandt *et al.*, 1946; Heller, 1953). Heller (1959*a*) found that tissue cultures may produce some natural chelating agent, possibly acidic, that keeps iron in solution at *p*H 6.0. Hildebrandt *et al.* (1946) reported that ferric tartrate was a better source of iron than was sulfate. Tartaric and citric acids were prominent among the acids that significantly increased the growth of tissue cultures (Hildebrandt *et al.*, 1954). Street (1957) found that the *p*H at which iron citrate or tartrate becomes unavailable is only 6.0. Street's suggestion that these metal ions could be chelated in the medium formed the basis of our study.

The Use of Fe-EDTA for Tissue Cultures

Our first experiments were directed toward establishing the optimal level of an Fe-EDTA complex in a slightly modified White's medium (the concentration of phosphate was twice that initially recommended by White). Growth of the

habituated tissue of *Daucus carota* was increased 250 per cent by the optimal concentration of iron chelate (FIGURE 1). This concentration of the EDTA alone was, as reported by Heller (1959), toxic to tissue, the iron alone was without effect, but the chelate was clearly stimulatory (TABLE 1). Heller and Richez (1959), using labeled iron, found that the addition of uncomplexed

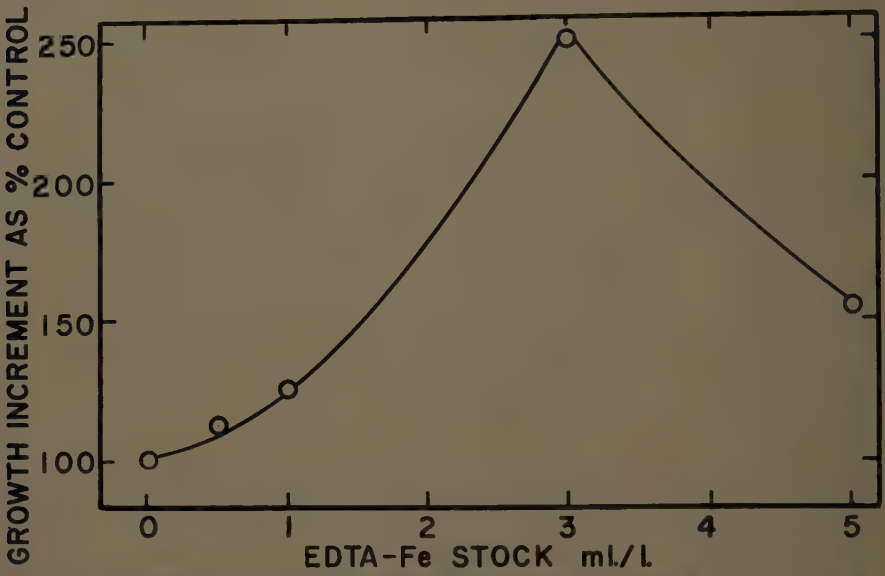

FIGURE 1. Effect of concentration of Fe-EDTA chelate on growth of *Daucus carota* habituated tissue cultures. White's medium at 24° C. for 21 days. Fe-EDTA chelate stock: Na_2EDTA, 800 mg./l.; $Fe_2(SO_4)_3$, 381 mg./l.

TABLE 1

EFFECT OF EDTA, Fe^{++}, AND AN EDTA-Fe CHELATE ON GROWTH OF *DAUCUS CAROTA* HABITUATED TISSUE CULTURE ON WHITE'S MEDIUM AT 24° C. FOR 21 DAYS

Additive*	Increase in fresh wt. (%)
None	162
EDTA	29
Fe	154
Fe-EDTA	328

* EDTA, 2.4 mg./l.; Fe^{++}, 1.14 mg./l.

EDTA appeared to increase the uptake of iron into plant tissues. Kordan (1959) has recently used chelated iron for culturing the juice vesicles of lemon.

The supposition that the complex is a cumulative "poison" when supplied continuously was discarded after a clone of tissue was grown for 18 successive two- to three-week transfers on medium containing this chelate: no dimunition of growth rate was noted. At the end of this period, a reciprocal experiment was set up in which some of these tissues were transferred back to standard

White's medium, while some from White's medium were placed on the medium containing the chelate. As TABLE 2 shows, those taken from White's grew at the rates characteristic of those on chelate medium, while those taken from chelated medium returned to their previous rates of growth.

TABLE 2

EIGHTEEN RECIPROCAL TRANSFERS OF *PARTHENOCISSUS TRICUSPIDATUS* CROWN GALL TISSUE CULTURE ON WHITE'S MEDIUM AND ON WHITE'S MEDIUM WITH FE-EDTA, AT 24° C. FOR 21 DAYS

Transfers on medium	Test medium	Increase in fresh wt. (%)
With Fe-EDTA	EDTA-Fe	355
With Fe-EDTA	White's	138
Alone	White's	150
Alone	EDTA-Fe	364

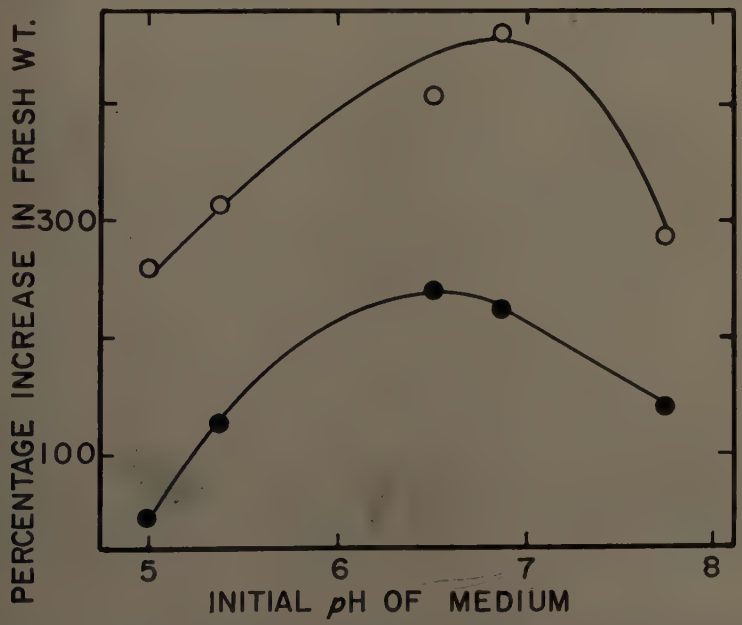

FIGURE 2. Influence of initial *p*H of medium on growth of *Daucus carota* habituated tissue cultures. *Open circles*, White's medium and Fe-EDTA; *filled circles*, White's medium. Growth at 24° C. for 14 days.

Buffering of Plant Tissue Culture Media

Since iron, when chelated, would not be limiting at neutral *p*H values, we examined the optimal *p*H for growth of a tissue culture. In these experiments the growth period was kept at two weeks, a period of time during which endogenous supplies of iron would be unlikely to become depleted even in unchelated media (cf. Heller, 1953). In media supplemented with Fe-EDTA and in unsupplemented media the optimal initial *p*H was close to neutrality (FIGURE 2). Here, as in previous experiments, chelated iron enhanced growth. The

results differ from those obtained by others, in that previously reported optima were closer to 6 than to 7. We attribute this difference to the unavailability of iron at elevated *p*H values when experiments are extended beyond a few weeks (Hildebrandt *et al.*, 1945).

Provasoli *et al.* (1957) reported that tris(hydroxymethyl)aminomethane (Tris) was an inert but effective buffer in culture media for marine algae. Its use for plant tissue cultures seemed indicated and, indeed, the addition of an experimentally determined amount of Tris (5×10^{-4} *M*) to White's medium supplemented with Fe-EDTA was a distinct improvement (TABLE 3). The revised formulas for White's medium containing Fe-EDTA and Tris at *p*H 7.0 permitted a fourfold increase in the growth of tissue culture (FIGURE 3). On occasion we have observed increases as great as fivefold, and in all experiments the physical appearance of the tissues was greatly improved. That such beneficial effects are not restricted to this one tissue is apparent from TABLE 4.

TABLE 3

EFFECT OF Fe-EDTA CHELATE AND TRIS BUFFER (5×10^{-4} *M*) ON GROWTH OF *PARTHENOCISSUS TRICUSPIDATUS* CROWN GALL TISSUE CULTURE AT 24° FOR 21 DAYS

White's medium	Initial *p*H	Increase in fresh wt. (%)
Alone	5.3	163
Alone	7.0	200
With Fe-EDTA	5.3	384
With Fe-EDTA	7.0	431
With Fe-EDTA + Tris	7.0	592

FIGURE 3. *Parthenocissus tricuspidatus* crown gall tissue cultures grown on White's medium containing (*left to right*) no additions, Fe-EDTA chelate, and Fe-EDTA chelate with Tris buffer, 5 weeks after inoculation.
Photograph by E. N. Mitchell.

Callus, habituated, and crown gall tissues of *Parthenocissus tricuspidatus* and habituated and crown gall tissues of *Helianthus annuus* grew better on the media containing both Fe-EDTA and Tris than on the media without buffer. In the absence of either supplement, growth was less.

Finally, it should be mentioned that the Fe-EDTA supplementation procedure has been in use in our laboratory for some time and also has been used in other laboratories with equally good results.

Discussion

There is no need to discuss either the theory upon which these studies were based or the data obtained. Plant tissue cultures can be grown faster and more easily on media containing chelated iron. Yet we know that iron is not the only metal ion needed in trace amounts, and future studies must be directed toward the elucidation of the requirements for zinc, manganese, cobalt, and other metals. Such work is in progress in our laboratory. Preliminary experiments with Hutner's No. 47 dry metal mix chelated with an organic salt of EDTA appear to give excellent results. Additional work is clearly indicated.

Table 4

Comparison of White's Medium and Fe-EDTA, and White's with Fe-EDTA and Tris Buffer (5×10^{-4} *M*) *p*H 7.0 on Growth of Tissue Cultures at 24° C. for 18 Days

Plant	Increase in fresh wt. (%)					
	Callus*		Habituated		Primary crown gall	
	Fe-EDTA	With Tris	Fe-EDTA	With Tris	Fe-EDTA	With Tris
P. tricuspidatus	52	91	112	330	220	485
H. annuus	69	77	150	210	186	294

* Medium contained naphthaleneacetic acid, 50 μg/l.

It should be emphasized that we are not certain that all of the growth responses of tissue cultures are due to the buffering of iron by EDTA. The chelating agent probably is complexing other metals that are in competition with iron, and such interactions would be very difficult to analyze.

The fact that Fe-EDTA increased the growth of crown gall tissues of both *Parthenocissus* and *Helianthus* is a further indication that its role is not that of an auxin (cf. Heath and Clark, 1956*a* and *b*). Both these tissue cultures are prototrophic for auxins and are, in fact, inhibited by these growth regulators. Moreover, there was no indication that EDTA would substitute for the auxin known to be required by callus tissues (cf. Klein, 1957).

Without evidence, we are inclined to think that at least some of the positive effects on the growth of tissue cultures of such complex natural additives as raw coconut milk may be due to their metal-chelating ability. The presence of organic acids in the supplements tends to reinforce this concept, and work along these lines might be profitable.

Summary

Standard media used for plant tissue cultures can be improved by the addition of chelated metal ions, alterations in the hydrogen-ion concentration, and adequate buffering with a metabolically inert reagent. Such modifications have resulted in the formation of a medium that permits growth, measured by fresh weight increments, to proceed at rates four to five times those obtained on standard media. It is, however, unlikely that these alterations have completely satisfied the requirements of the tissues for metal ions. The increased rates of growth are still below those known to be within the capacity of plant cells.

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Part III. The Medical Applications of Chelating Agents

THE INFLUENCE OF THE PHYSIOLOGIC DISPOSITION OF CHELATES ON THEIR USE IN MEDICINE

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Introduction

Most drugs in use today have come to the attention of the physician by way of empirical observations or chemical analogy with known active compounds. It is, however, more satisfying to the research worker to predict the potential usefulness of an untested class of compounds by a knowledge of their physicochemical properties. The chelates are such compounds. Of course, in the last analysis, the method that requires the least intelligence but is ultimately the most trustworthy, namely, trial-and-error, must be employed to determine the true worth of a chemical agent in clinical medicine.

The concept of chelation, which has been developed from careful physicochemical studies of relatively controlled systems, need not be reviewed here. We propose to limit ourselves to a discussion of some N-carboxy chelates and to review the fate of these agents in the body, with a view to correlating their physicochemical properties and physiologic disposition with their potential therapeutic value.

Chelate action in the living organism cannot always be explained by the simple assumption that a metal vital to some important enzyme system is removed by the agent. One need only cite the remarkable observation that adding ethylenediaminetetraacetic acid (EDTA) to an enzyme-free system of iron-ascorbic acid markedly speeds up the *p*-hydroxylation of many aromatic compounds, an effect which one might expect to require the presence of an iron-dependent natural enzyme.

Since so many of life's processes are fundamentally enzymatic, and since enzymes may be considered substances that alter the dynamics and specificity of relatively simple spontaneous chemical reactions involving metals and organic compounds, it is no surprise that agents that can bind and alter the disposition of metals may be considered for an extremely wide variety of uses. In fact, there have been reports on the possible application of N-carboxy chelates to almost every basic type of disease to which man falls victim. There are literature references to the application of the chelate concept to problems of allergy, cancer, infection, poisoning, degenerative diseases, inflammatory diseases, errors in metabolism, deficiency diseases—in fact, every chapter heading found in a standard textbook of medicine.

Fundamental to all these potential uses of chelates is an understanding of what happens to them and to the metals they influence in the body.

Absorption

The factors determining the absorption of chelates and of organic complexes in general are poorly understood. Certainly, the solubility of EDTA, for

example, in aqueous solution at physiologic *p*H is high, and the molecular size is not prohibitively large. Yet we know that little if any of orally administered EDTA is absorbed. The relationship of the absorbability of this class of compounds to the lipid-water solubility ratio and the *p*K characteristics of the compounds remains to be determined. There is evidence that some of the newer N-carboxy chelates are absorbed from the gastrointestinal tract considerably better than is EDTA.

The absorbability of different metal complexes of the same ligand may be quite different. Iron complex absorption is sometimes sufficient to color grossly serum, and orally administered Cu-EDTA results in the gross appearance of the characteristic color of that complex in the urine. Yet orally administered Na-EDTA or Ca-EDTA is notoriously unsatisfactory for introducing the chelate into the body: not only is absorption poor, but diarrhea is common. The diarrhea has been compared to that resulting from such metal-deficiency states as zinc deficiency in animals. However, there is no proof available as yet that this intolerance results from an induced deficiency state.

Absorption of an orally ingested chelate is not necessary to influence the normal metabolic fate of calcium or other metals that may be found in the gastrointestinal tract. Considerable alteration in the normal cycle of gastrointestinal secretion and reabsorption of some metals may be induced by the fecal excretion of an unabsorbed complex.

Thus far most therapeutic applications of chelating agents have been made by slow intravenous infusion. The slowness is especially essential when monovalent salts of a free chelating agent are administered if a precipitous drop in plasma calcium levels is to be avoided. Intramuscular injection of EDTA has proved to be too painful for extensive investigation in man, although the magnesium complex, studied primarily for its hypotensive effect, is said to be well tolerated when administered by this route.

Because of absorption characteristics, several precautions are in order, in the interpretation of the usual type of LD_{50} data, as regards free metal versus metal chelate toxicity. For example, the subcutaneous injection of some simple salts of rare earths results in the temporarily harmless precipitation of much of the metal at the site of injection. In contrast, a similar injection of the chelated metal may result in more rapid absorption and distribution, the systemic effects of the rare earth becoming more rapidly apparent. The same comparison of simple salt and chelate, each given by the intravenous route, almost always shows the free metal to be much more toxic. Because of the altered distribution and possibly delayed liberation of toxic metal from a complex or a site of injection, animals used in "acute" LD_{50} studies of metals and their chelates should be observed for at least two or three weeks before their final status is recorded. Even intraperitoneal injection of some metal complexes has been shown to result in the localization of significant amounts of metal in the peritoneal lining, from which site further absorption may take place quite slowly and influence toxicity accordingly.

The absorption of topically applied chelates, as of other organic compounds, probably varies with the nature of the base into which it is formulated. Little if any EDTA, topically applied, gets into the blood stream, although no doubt it can react with some cations at or near the surface. Chelate binding with

surface metals may alter the ordinary skin reaction to the metal, as it clearly does in nickel-sensitive patients. Rates of binding equilibrium may become very important; this appears to be the case in chrome sensitivity, where the protective value of EDTA cream is considerably less obvious. It is not beyond possibility that mobilization of a metal by chelate complexing can occur without inactivating the metal's sensitizing properties, in which case a sensitivity reaction conceivably would be aggravated.

Binding in Blood

When a trace metal complex is identified in blood it is difficult to be sure how much, if any, is due to penetration of the chelating agent into tissues containing deposits of the trace metal and return of the complex to blood, rather than due to direct complexing with trace metal already in the blood itself. All other factors being equal, the *in vitro* stability constants of various metals for a given chelating agent reflect the degree to which a metal will compete successfully with other metals. This accounts for the almost complete binding of chelating agents to calcium, in preference to magnesium. On the other hand, even trace amounts of most heavy metals will replace calcium, since their higher stability constants represent a capacity to bind that is often several orders of magnitude greater than that of calcium. One must, however, consider not only the competition of metals for a chelating agent but also the competition of an administered chelating agent with naturally occurring binding substances for a given metal. One might expect a simple equilibrium of competitive binding or, practically speaking, saturation of the dominant ligand, before any metal is available for binding with the weaker ligand. However, in some systems the equilibrium rate apparently is so slow that for practical purposes the metal stays largely with the ligand with which it is first complexed. In such situations an administered chelate may not be able either to remove or to contribute a given metal to its competitive natural complexing agents. Since these equilibrium rates can range from very fast to extremely slow, the action of the chelate can be equally varied.

In the living animal, chelates such as EDTA cannot be given in quantities large enough to bind significant amounts of magnesium, since a fall in free calcium concentration to levels incompatible with life would result. However, there are some interesting interrelationships between the concentrations of calcium and magnesium in blood *in vivo*, and these are no doubt altered by chelating calcium. Calcium concentration in several respects has reciprocal effects to potassium as regards cardiac action. Quite possibly the hypotensive response to administered Mg-EDTA is just as much a function of the resulting fall in free calcium as of the rise in magnesium concentration in blood. *In vitro* studies permitting the chelation of both calcium and magnesium in blood have demonstrated the reversible inactivation of some clotting enzymes that presumably require free magnesium.

Strontium metabolism also is linked intimately with that of calcium. The ratio of circulating free calcium to strontium may be significantly altered by the administration of EDTA, which combines with calcium much more readily than with strontium.

Since *p*H markedly influences complex formation, the properties of a chelate

at *p*H 7.35 are of great importance. In general, the capacity to bind with heavy metals increases with increasing *p*H. It is sometimes forgotten that an actual change in some ligands such as protonation or deprotonation may take place with change in *p*H, and these altered ligands may have significantly different metal-binding properties. Chelates such as EDTA, cyclohexyl *trans*-1,2-diaminetetraacetic acid (CDTA), and diethylenetriaminepentaacetic acid (DTPA) are essentially 100 per cent calcium-complexed at the physiological *p*H range of 7.1 to 7.4. But this is not true of all chelates. Compounds with incomplete binding, especially of calcium, at and near this *p*H range, are of particular interest (FIGURE 1). One can imagine a cycle involving an absorbable metal complex that forms at the *p*H of the intestine (8.3), is absorbed, and then is dissociated at *p*H 7.35 in the blood; the free chelating agent then may be secreted via the bile into the intestine, where it may complex again with an otherwise unabsorbable metal, and the cycle is repeated. Rela-

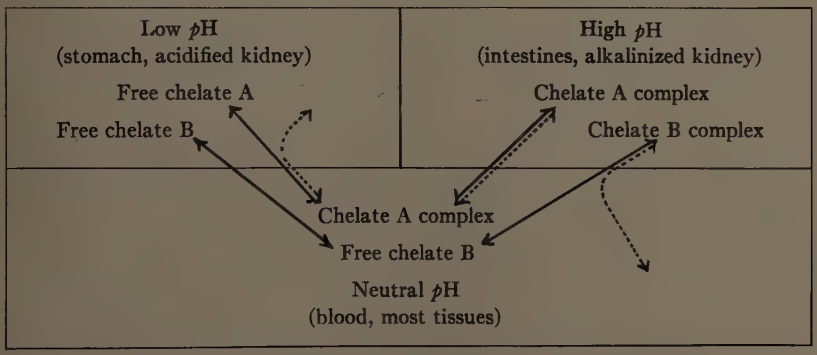

FIGURE 1. Schematic presentation of the effect of movement of chelates between body pools of different *p*H. Chelate A represents an agent essentially completely complexed at neutral *p*H, in contrast to chelate B, which exists to a significant degree as the free chelating agent at *p*H 7.

tively small amounts of such a chelating agent may account for the absorption of many times its molar equivalent of a metal.

Many organic compounds, particularly aromatic drugs, circulate to a large extent bound to plasma proteins. This binding may or may not involve a metal bridge. Should a chelating agent become so bound, no doubt it would influence the equilibriums of reactions in which the ligand is involved.

Excretion via Kidney

The renal disposition of bound or free chelate depends on (1) chelate properties at the *p*H of blood and urine, (2) *p*H of nephron structures at the time the chelate is being eliminated, and (3) natural competitive binding components of renal cells and urine. These factors are particularly important as regards the toxicity of chelate complexes. Chelates "detoxify" not only by binding but by the renal elimination of the bound material. Yet chelates of metals such as lead and bismuth, although markedly less toxic than the unbound metals, are not harmless in the body. When these heavy metal complexes are administered, even with excess chelating agent, some of the heavy metal escapes the chelate, and this escape may occur, at least in part, in the kidney.

Efforts to produce useful radiopaque agents by chelating heavy metals are stymied because of this escape.

Reports of kidney toxicity in man due to chelating agents largely have involved patients being treated for heavy-metal poisoning. At least a part of the observed toxicity may be due to the mobilization of the toxic metal and its partial deposition in the kidney rather than to chelating agent toxicity per se.

Attempts to prevent or dissolve calcific renal calculi with N-carboxy chelating agents thus far have not been very successful. Retrograde irrigation has proved to be too irritating, or ineffective, or both. Systemic treatment has followed two ideas:

It was reasoned that, through administration of EDTA and acid ash diet or NH_4Cl, the EDTA in the resulting acid urine would be partially uncomplexed and therefore might pick up urinary calcium. It is clear, however, that the chelate is incompletely complexed at the acid *p*H, because it is incapable of binding any more calcium at that *p*H. Only if something should happen in the urine reservoir to increase *p*H subsequently, might the complexing of additional calcium be expected. For example, urea-splitting organisms in chronic pyelonephritis might induce such a situation. The theory, however, has not been applied successfully.

A second approach is the administration of a chelating agent that does not bind completely with calcium at *p*H 7.35 and will bind more calcium as it is excreted with an *alkalinized* urine. Since chronic therapy is required, the agent not only would have to have the proper *p*H dissociation properties but also would have to be orally absorbed and nontoxic. Even such a compound might not succeed in dissolving or preventing calculi. The concentration of calcium phosphate in urine is several times greater than its concentration in fully saturated simple aqueous solution. The nature of the urinary organic matrix that normally keeps urinary calcium in solution may have far more influence than the addition of exogenous chelates with a theoretical capacity to bind calcium in simplified hypothetical systems. To date there is no effective way of treating renal calculi by the administration of chelating agents.

Some metals are excreted ordinarily by the kidney only to a very limited extent. Iron, for example, normally appears in the urine in insignificant quantities. However, if an iron chelate is administered or an appropriate calcium chelate is given to patients with abnormal iron deposits, the iron chelate presented to the kidney is largely excreted. This is also clearly true of other metals that form more stable complexes than calcium, such as lead and plutonium. In contrast, calcium is bound in preference to strontium by chelating agents such as EDTA, CDTA, and DTPA, so that renal strontium excretion is not increased by these agents. In fact, administration of Na-EDTA, which binds with plasma calcium, temporarily reduces the total *free* calcium presented to the kidney for excretion, and strontium output actually may go down, since its excretion is a function of the amount of free calcium excreted. This, however, need not be the case if one can find a chelating agent whose stability constant for strontium at least approaches, if not surpasses, that for calcium. Evidence for this concept is seen in preliminary comparisons of EDTA and Chel ME in this connection.

Tissue Disposition

The importance of the capacity of a metal chelate to remain complexed under physiological conditions lies not only in the difference in renal handling of metal chelate and that of free metal but also in the mobilization of metals from sites of tissue deposits. Here again the stability constant of a given metal in relation to that of calcium at a given site is of fundamental importance. One might guess that, if the stability constant for a given metal is much higher than for calcium, it wouldn't matter very much how much higher it was. Can the better than 1000:1 preference of EDTA for plutonium or iron over calcium be improved upon? DTPA shows about a 100,000:1 ratio. Comparison of the effectiveness of EDTA versus DTPA demonstrates that DTPA has a clinically significant superiority over EDTA for these and other metals.

While the stability constants for most metals line up in the same order for different chelating agents, the actual constants are not quantitatively parallel, and in some instances the order may change with changing *p*H. It is intriguing that natural complexes such as heme and B_{12} regularly pick their characteristic metal out of the body pool. Referring to these highly selective metal complexes as metalloenzymes as opposed to reversible metal chelates does not explain the mechanism of this highly important selectivity.

Selective action may be achieved through penetrability of an organic molecule into various tissues. Modifications such as cyclic chelates and ethers may be expected to have distribution properties quite different from "the usual" simple aliphatic chelates. With increasing knowledge concerning membrane transport and other mechanisms, compounds may be designed to penetrate relatively specific tissues as inactive moieties, and may be metabolized into forms capable of chelating metals at sites otherwise inaccessible.

The fate of metal deposits in different tissues depends in great part upon the nature of the binding in each tissue. Some metals injected as chelate complexes into the blood stream or nearly simultaneously with a calcium chelate are rapidly and almost completely eliminated. If the free metal has had a relatively few minutes in the body so that some tissue deposition has taken place, chelate administration may not be quite so effective in causing complete renal excretion of the metal, but it still can cause a marked increase over that in a control without chelate. If, however, the metal has become incorporated deeply into bone, the chelate's ability to remove the metal may be considerably diminished. For example, DTPA will greatly increase the daily urinary elimination of several heavy metals. After prolonged administration, however, the soft-tissue pools of the metal may become depleted more rapidly than the bone deposits, so that metal excretion does not continue at a high level even when there is still a considerable metal reservoir in bone. In the case of some toxic metals the removal from soft tissues is the primary objective of therapy. With others, particularly radioactive elements, elimination of bone deposits is equally vital. Intermittent rather than repeated frequent therapy with chelates may prove the most desirable, since it may permit a partial re-establishment of bone-soft tissue distribution between courses of therapy with the best return per unit of chelate administered.

It is interesting to observe the pattern of heavy-metal excretion following a single dose of chelate. Studies with labeled Ca-EDTA and DTPA have shown that over 90 per cent of the chelate is excreted in 4 hours, and essentially all is in the urine in 24 hours. Nevertheless, lead and plutonium excretion is clearly greater for several days after a dose of chelate than during the control period. This may be due to mobilization of the metal by the chelate from previously inaccessible sites to tissues from which the metal can more readily reach the kidney. These sites may include the kidney parenchyma itself. On the other hand, the phenomenon may result from a different renal excretion rate for Pu-DTPA from that for Ca-DTPA.

It is almost, but not quite always, true that a metal chelate is less toxic than the free metal. Conceivably, a chelate more effectively may transport a metal to a site where its toxic activity is particularly great. More generally, however, chelate binding prevents a metal from exerting its usual toxicity.

Biliary Secretion

It has been shown with some labeled metal chelates that distinct quantities of complex can be recovered from the stool after parenteral administration. This is clear evidence of complex secretion into the gastrointestinal tract, probably by way of the bile. Some complexes of a given chelate may take part in a cycle of biliary excretion and reabsorption, while others may not.

No doubt, the future will bring new chelating agents that are significantly metabolized rather than excreted unchanged via the kidney. Some agents may become effective chelaters only after metabolic conversion. Such conversion is likely to occur in the liver. The metabolic products of some organic acids are known to be secreted quantitatively into the bile and to reach the blood stream only by subsequent reabsorption from the gastrointestinal tract. Very interesting and novel effects may result from chelating agents with this pattern of disposition (FIGURE 2).

Discussion

Although our discussion has concentrated on the physiologic disposition of chelates, I have had occasion to mention some of the clinical applications of these agents. Calcium chelates and, particularly, Ca-DTPA, already hold a place of importance in the treatment of heavy-metal toxicity involving iron, lead, plutonium, and other metals and fission products. Skin sensitivity to metals also has been controlled with chelates. N-carboxy chelating agent therapy for removal of calcium has proved promising in calcinosis, scleroderma, and problems of arrhythmias, but has been disappointing as regards renal calculi. Early results in calcific atherosclerosis clearly point to the desirability of further study, as does the influence of chelates on certain metal-dependent toxic enzymes and pathological microorganisms. A similar rationale has led to the study of the cytostatic action of chelates and the chelating capacity of known cytostatic agents. For example, DTPA was found to have distinctly inhibiting effects in some experimental tumors, although the promising initial results were not upheld upon more critical study. It remains to be determined how chelate therapy may augment the cytostatic effects of known anticancer

agents. Calcium binding *in vitro* has proved its value in blood preservation and related problems encountered in the hematology laboratory. The magnesium-calcium balance probably is important here as well as in the treatment of hypertension with magnesium chelate cited before.

The low acute toxicity of chelated heavy metals has inspired rather extensive studies of these complexes as radiopaque agents. While excellent radiographic properties were found even *in vivo*, no chelate is available that can hold complexed 100 per cent of its heavy metal against the onslaught of the body's competitive binding agents and enzymes before being excreted. Until such a chelate is available, the tissue deposition of even relatively small amounts

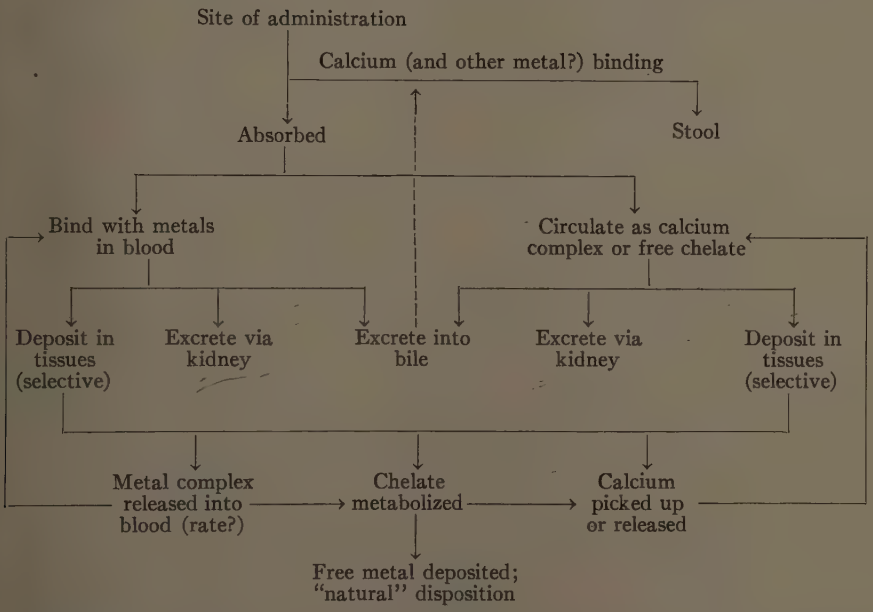

FIGURE 2. Summary of possible pathways of chelating agents and their calcium or other metal complexes in the body.

of "escaped" heavy metal precludes the use of these agents for the usual diagnostic procedures.

The use of chelates as vehicles carrying needed metals to desired sites is now under investigation along several lines. The seemingly paradoxical idea of using a chelate to treat calcium deficiency appears less paradoxical when one observes that animals deficient in calcium are clearly more capable of taking calcium from the chelate than are normal "calcium-saturated" subjects. A similar hope exists, not yet clearly realized, concerning iron chelates, which, unlike other forms of iron available for the treatment of anemias, can be at least partly excreted by the kidney if an excess is given. There is no doubt that chelated iron can be utilized by iron-deficient animals. Evaluation of oral iron chelates and, especially, the weaker complexes such as iron glycine requires careful attention to the *p*H environment to which the complex is

subject. For example, good absorption and utilization of weak iron chelates has been noted in gastrectomized patients and others with poor gastric acidity. We suspect, however, that these weak complexes may be dissociated at the *p*H of normal gastric juice, and the resulting free iron in solution with the normal products of digestive proteolysis will be indistinguishable from iron administered as the ordinary inorganic salt. Much remains to be done to determine the potential value of stronger iron chelates as oral or parenteral therapeutic agents.

Chelates as carriers have had some limited success in transporting radioactive elements to tumor sites. Ascites tumors, myeloma, and related malignancies have responded to such therapy.

It is clear that many directions are still open for the clinical application of chelates. Further investigations no doubt will point out which of these directions deserve our greatest attention.

STUDIES OF THE EFFECT OF CHELATING AGENTS IN MAN*

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The availability of agents that bind metals *in vitro* and *in vivo* irreversibly under certain conditions offer a unique opportunity to study metal metabolism in experimental animals and in man. The binding of different metals to the chelate and the tightness of the bond depend on the stability constant of the metal chelate and on the *p*H at which the binding takes place. Calcium is tightly bound by ethylenediaminetetraacetic acid (EDTA) in the alkaline or neutral *p*H range (stability constant: log K = 10.06). The experimental inactivation of calcium *in vivo* is an interesting tool in studies of the mechanism of calcium homeostasis. The binding of calcium by chelating agents in plasma and the lack of the availability of this element for physiological function call compensatory forces into play to maintain calcium homeostasis which, in turn, maintains the serum calcium at the level required for the normal function of the organism. The ionic calcium that is bound and transformed into a Ca-EDTA complex is rapidly excreted by the kidney.

This presentation is a short summary of work carried out with chelating agents in this laboratory in the past several years. Extensive studies have been carried out on the effect of chelating agents on calcium metabolism in normocalcemic and hypercalcemic patients at this institution in the past.[1,2] EDTA† was administered as the sodium or calcium salt by the intravenous, intramuscular, and oral routes. The effect of EDTA was also investigated on the removal of radioactive isotopes[3-5] from man, such as calcium (Ca^{45}), lanthanum (La^{140}), yttrium (Y^{90}) and zinc (Zn^{65}). The availability of a series of new chelating agents‡ has permitted comparative studies of the metabolism of these chelates in experimental animals and man. The toxicity and the tolerance of these agents was studied. These investigations led to the application of these chelating agents in physiological studies and in studies of decontamination, especially on the removal of yttrium.[5] The metabolism of tracer doses of several yttrium chelates was studied at first in man in order to obtain information on the magnitude of excretion of yttrium administered in the form of different chelates.[6] This information seemed of importance for the selection of specific chelating agents as decontaminating agents. The effect of some of the newer chelating agents on the removal of yttrium was compared with that obtained with the conventionally used chelate EDTA. The great affinity of one of these agents, diethylenetriaminepentaacetic acid (DTPA), for yttrium was used in studies of renal clearances and of the space of distribution of this chelating agent.[7] Labeling of chelating agents with C^{14} carried out in our laboratory§ led to studies of the excretion of tracer doses of labeled chelates

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† Supplied by Riker's Laboratory, Inc., Los Angeles, Calif.

‡ Supplied by the Geigy Pharmaceutical Company, Ardsley, N. Y.

§ Synthesized by Harry Kroll, Montefiore Hospital, New York, N. Y.

in man. Studies carried out with EDTA, DTPA, and bis(2-aminoethyl) ether tetraacetic acid (BAETA) will be described.

Results

TABLE 1 shows an example of the effect of Na-EDTA on urinary calcium excretion in normocalcemic persons. The urinary calcium excretion increases markedly on the day of the slow intravenous infusion of the 4 gm. Na-EDTA given over a period of 4 hours. This rather slow administration of the chelat-

TABLE 1
EFFECT OF Na-EDTA ON URINARY CALCIUM EXCRETION IN NORMOCALCEMIC MAN

Patient	Urinary calcium, mg./day			Excess excretion† (%)
	Before	During*	After	
1	75	321	61	57
2	89	314	107	63
3	26	333	40	71
4	14	317	10	70

* Four gm. Na-EDTA infused in 500 ml. 5 per cent glucose in water in 4 hours.
† Per cent of theoretically expected excess excretion. One gm. Na-EDTA binds 108 mg. calcium.

TABLE 2
EFFECT OF Ca-EDTA ON URINARY CALCIUM EXCRETION IN NORMOCALCEMIC MAN

Patient	Urinary calcium, mg./day			Excess excretion† (%)
	Before	During*	After	
1	69	394	61	82
2	108	365	118	79
3	20	367	35	88
4	18	313	30	75

* Intravenous infusion of 4 gm. Ca-EDTA in 4 hours.
† Per cent of theoretically expected excess excretion: 1 gm. Ca-EDTA contains 98 mg. calcium.

ing agent decreased only slightly and temporarily the serum calcium level. The excess calcium excretion in urine was calculated in per cent of the expected binding of calcium by the amount of the chelating agent infused. The lack of change in the serum calcium level and the concomitant increase of the urinary calcium excretion indicated rapid replacement of the chelated calcium in plasma by calcium released from the skeleton. The excess calcium excretion did not approach the expected excretion of 100 per cent but ranged from 57 to 70 per cent. This finding may be due to binding of other trace metals by EDTA or to dissociation of the calcium chelate induced by *p*H changes in the kidney and tubular reabsorption of ionic calcium.[8]

TABLE 2 shows the effect of the slow infusion of chelated calcium (Ca-EDTA) on the excretion of urinary calcium. Here again the urinary excretion of

calcium is markedly increased on the day of the infusion of the calcium chelate and an average of 81 per cent of the expected excretion is recovered in the urine.

The rapidity with which the excess calcium is excreted by the kidney following the infusion of Na-EDTA is shown in TABLE 3. Approximately 60 to 80 per cent of the excess calcium is excreted in 4 hours and a small percentage within 4 and 8 hours; occasionally some of the excess calcium is still excreted between the eighth and twenty-fourth hour.

Since the excess calcium excretion following the infusion of Na-EDTA or Ca-EDTA did not approximate the expected excretion, tracer doses of Ca^{14}-

TABLE 3

DISTRIBUTION OF THE EXCESS EXCRETION OF CALCIUM WITH TIME FOLLOWING THE INFUSIONS OF NA-EDTA AND CA-EDTA

Chelate	Excretion of excess calcium (hours)*		Total excess excretion (%)
	0–4	4–8	
Na-EDTA	80	20	58
Ca-EDTA	56	44	88

* Hours after the start of the chelate infusion. Four gm. Na-EDTA or Ca-EDTA were infused. Excess excretion = percentage of theoretically expected excess excretion. One gm. Na-EDTA binds 108 mg. Ca^{++}. One gm. Ca-EDTA contains 98 mg. Ca^{++}.

TABLE 4

EXCRETION OF TRACER DOSES OF C^{14}-EDTA IN MAN

Patient	Per cent of administered dose excreted in hours			
	0–4	4–8	8–24	Total 0–24
1	62	20	16	98
2	64	20	18	102
3	64	22	15	99

labeled EDTA were studied in man. TABLE 4 shows that approximately 100 per cent of the labeled chelate is excreted in 24 hours, an average of 60 per cent being excreted in the first 4 hours, 20 per cent between the fourth and eighth hours and the rest between the sixteenth and twenty-fourth hours. These results are in agreement with data previously reported by Foreman *et al.*,[9] who have studied the excretion of C^{14}-labeled EDTA in man. However, these investigators infused the C^{14}-EDTA tracer together with an excess of 2-gm. carrier Ca-EDTA, which may enhance the excretion of the tracer.

Changes of the urinary calcium excretion by Na-EDTA were correlated with changes of urinary phosphorus excretion in an attempt to investigate whether the prompt release of calcium to maintain the serum calcium at normal levels in response to the binding of calcium in plasma is due to parathyroid stimulation. In some instances, the urinary phosphorus excretion increased on the

day of the Na-EDTA infusion, and the serum phosphorus excretion decreased. However, this finding was not consistent (TABLE 5). Therefore the mechanism of action by which Na-EDTA maintains calcium homeostasis in terms of stimulation of the parathyroid gland is not clearly defined. The rise of urinary phosphorus excretion is not a clear-cut indication for parathyroid stimulation, since this increase may well be due to removal of phosphorus from bone concomitant with the removal of calcium. The infusion of Ca-EDTA was not accompanied by any particular change in urinary phosphorus excretion or of the serum phosphorus level (TABLE 5).

The effect of infusions of calcium as the gluconate on the serum calcium level and urinary calcium excretion of man has been extensively investigated at this institution in the past.[10-13] The retention of the infused calcium was found to

TABLE 5
EFFECT OF Na-EDTA* AND OF Ca-EDTA* ON PHOSPHORUS METABOLISM

Patient	Chelate	Urinary phosphorus, mg./day			Serum phosphorus, mg. % (hour)	
		Before EDTA	Day of EDTA	After EDTA	0	4
1	Na-EDTA	313	683	295	3.7	5.5
2	Na-EDTA	196	388	446	2.0	—‡
3	Na-EDTA	577	639	443	3.9	5.2
4	Ca-EDTA	604	499	454	3.5	3.7
5	Ca-EDTA	369	409	434	3.9	3.6
	Ca-EDTA	384	322	379	3.4	3.4
6	Ca-EDTA†	293	262	310	—	—

* Four gm. were injected intravenously in 4 hours.
† Two gm. were injected intravenously in 4 hours.
‡ Hemolyzed.

depend on the calcium metabolism of the individual studied and was significantly lower in persons with conditions of calcium loss[14] and higher in those with a tendency to calcium retention[15,16] than in normals. In contrast, the percentage of calcium excreted following the infusion of either Na-EDTA or Ca-EDTA, the excess calcium excretion, was similar in a large number of patients, although they were in different metabolic states. Examples of the comparative excess excretion following the infusions of Na-EDTA, Ca-EDTA, and calcium gluconate are shown in TABLE 6.

A study was performed to investigate whether Na-EDTA removes calcium from bone. This was done by administering the chelating agent to persons who had previously received radioactive calcium. Since 99 per cent of the body calcium is located in bone, the removal of Ca^{45} injected two weeks prior to the administration of Na-EDTA is most likely due to removal of this bone-seeking radioisotope from the skeleton by the chelating agent. FIGURE 1 shows

TABLE 6

EXCESS CALCIUM EXCRETION FOLLOWING THE INFUSION OF NA-EDTA, CA-EDTA AND CALCIUM GLUCONATE

Patient	Excess calcium excretion (%)		
	Na-EDTA*	Ca-EDTA*	Calcium gluconate
1	72	75	7
2	71	88	30

* Percentage of theoretically expected excess excretion. Four gm. Na-EDTA, 4 gm. Ca-EDTA, and 469 mg. calcium as the gluconate, respectively, were infused in 4 hours in 5 per cent glucose in water.

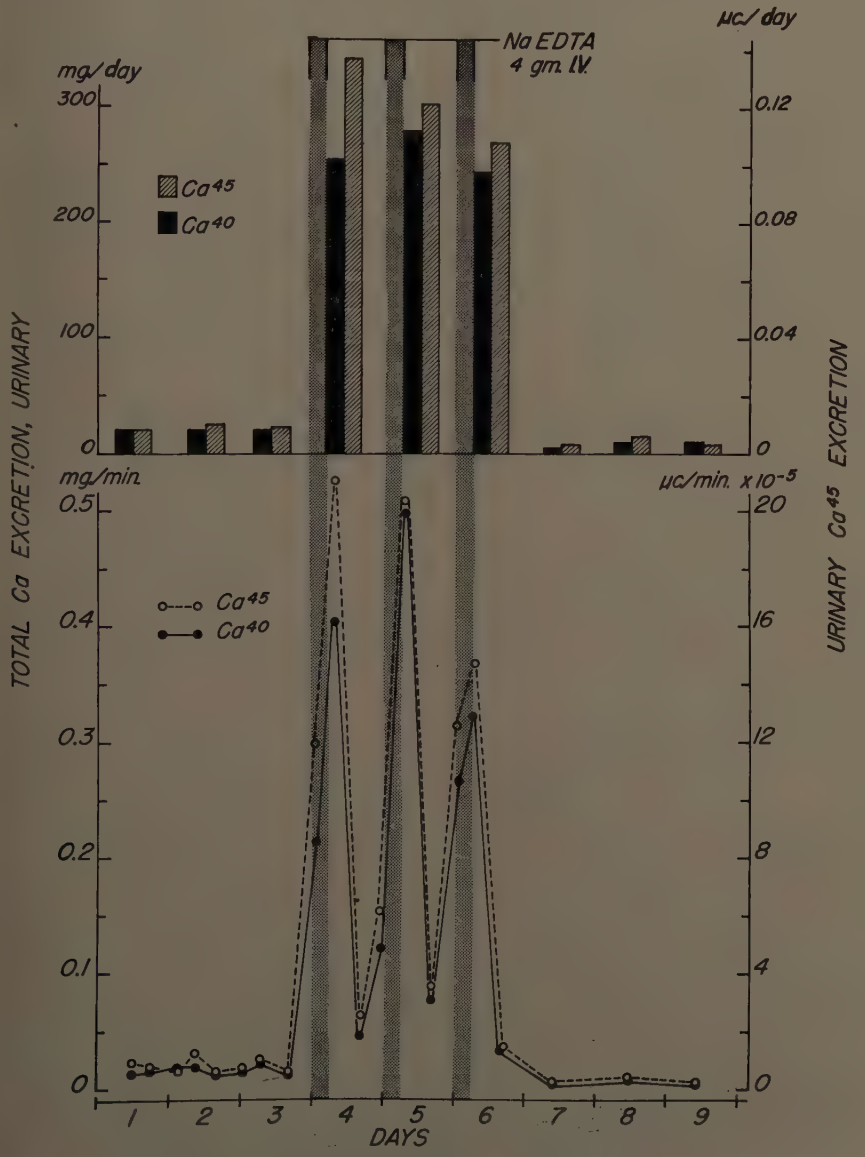

FIGURE 1. (Reproduced by permission of Bellin and Laszlo.[3])

a considerable increase of excretion of calcium and of Ca^{45} on the days of the Na-EDTA infusions. In spite of this marked change, the ratio of excretion of Ca^{45}/calcium, the specific activity (per cent dose Ca^{45}/100 mg. Ca) remained similar on the days before, during, and after the infusion of Na-EDTA. This constancy may indicate that Ca^{45} has been removed from accessible areas of the skeleton in which it had been deposited previously. Since most of the retained Ca^{45} was undoubtedly deposited in bone by the time the chelating

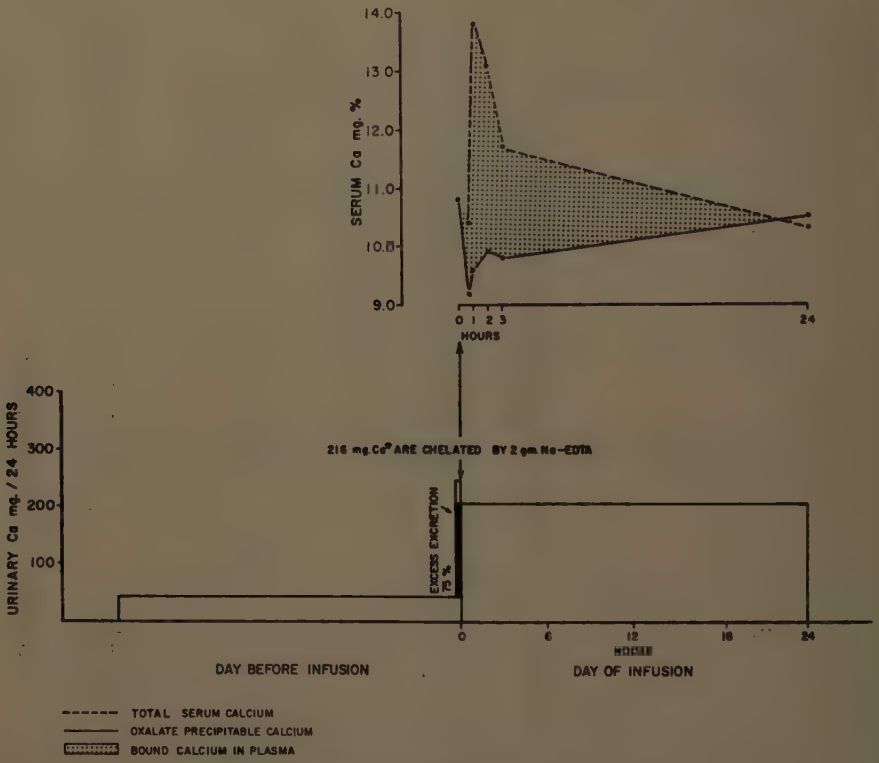

FIGURE 2. (Reproduced by permission of Spencer *et al.*[2])

agent was injected, EDTA removed stable *and* radioactive calcium from bone to restore calcium homeostasis in order to replace the bound calcium in plasma.[3]

The efficiency of the mechanism that controls calcium homeostasis was then tested by infusing Na-EDTA more rapidly to patients with normal serum calcium levels. FIGURE 2 shows the results of such a study. The serum calcium was determined by oxalate precipitation representing the ionized and, in part, the protein-bound calcium, as well as by oxalate precipitation of ashed sera representing the total calcium, which consists of ionized, protein-bound, and chelated calcium. Following the rapid infusions of 2 gm. Na-EDTA (given over 40 min.), the oxalate precipitable serum calcium was decreased only temporarily and was restored to normal levels within a few hours. The

influx of calcium from bone into plasma to replace the bound calcium must have been rapid, indicating a very effective mechanism for the maintenance of calcium homeostasis, since the total calcium rose sharply to 13.8 mg. per cent when the oxalate precipitable calcium was 9.2 mg. per cent. The total plasma calcium represented by the shaded areas decreases as it is excreted as excess calcium in the urine: 200 mg. calcium were excreted on the day of the Na-EDTA infusion as compared to the calcium excretion of 44 mg. on the day before the chelate infusion, the excess calcium excretion corresponding to 75 per cent of the binding power of the infused Na-EDTA.[2] The influx of calcium from the body stores into the circulation in response to the rapid binding of the ionized plasma calcium became evident as elevation of the total serum calcium which was hardly demonstrable when the chelating agent was infused at a slow rate to persons with normal serum calcium levels in whom the binding of calcium was slower and the influx of calcium into the plasma and the excretion of chelated calcium by the kidneys kept balance.

Since Na-EDTA did not appreciably decrease the serum calcium level in normocalcemic persons and was shown to invoke a compensatory release of calcium from the skeleton, EDTA was thought to be not useful for reducing the serum calcium level in patients with hypercalcemia, which is frequently caused by pathologic states of bone destruction. However, it was assumed that this decrease may be achieved without invoking the compensatory release of bone calcium if Na-EDTA is given in amounts sufficient to bind the excess calcium already present over and above the normal level in the circulation and to lower the serum calcium just to, but not below, the normocalcemic range. It was also assumed that the excess calcium excretion in urine would be much lower than in normocalcemic persons, since the calcium present in the circulation in excess of the normal level would be bound by Na-EDTA and additional calcium would not be released from the skeleton.

Studies were then carried out with Na-EDTA in patients with hypercalcemia. For the reasons stated above, a decrease of the high serum calcium level (oxalate precipitable) was anticipated, and only a small excess of urinary calcium excretion, or no excess at all, was expected if the serum calcium level would not be decreased below normal.

Table 7 shows the effect of Na-EDTA in patients with hypercalcemia. Examples of the effect of slow as well as of the rapid infusions are shown. Following the rapid infusion of 4 gm. Na-EDTA given over 8 to 15 min., there was a definite decrease of the serum calcium level (oxalate precipitable) in all patients tested. However, the serum calcium level returned rather rapidly to hypercalcemic preinfusion levels, due to the active process of metastatic bone disease. The excess urinary calcium excretion, however, was lower in all hypercalcemic patients who, at the same time, were hypercalciuric, than in those with serum calcium levels in the normal range. This was due to the fact that a large amount of ionic calcium was already present in the circulation which was bound by EDTA and was excreted as the calcium chelate.[2]

EDTA given by the intramuscular route as the calcium salt is well absorbed. Figure 3 shows that the urinary calcium excretion of each patient who received Ca-EDTA by this route increased to a similar extent as following the intravenous route. Therefore the excess calcium excretion following the intra-

venous and intramuscular route of EDTA compared well. However, intramuscular Ca-EDTA injections cause pain in local tissues that can be alleviated only partially by the admixture of procain to the calcium chelate solution.

Studies carried out with orally administered Ca-EDTA and Na-EDTA have

TABLE 7

EFFECT OF SLOW AND RAPID INFUSIONS OF NA-EDTA IN HYPERCALCEMIC PATIENTS

	Day before	Day of infusion	Day after	Excess Ca excretion (%)
	Slow infusion*			
Urinary Ca, mg./day	220	420	215	30
Serum Ca, mg. %	17.4	—	11.4	—
	Rapid infusion†			
Urinary Ca, mg./day	372	482	300	26
Serum Ca, mg. %	14.5	—	12.8, 12.3, 12.9, 12.7, 14.0‡	—

* Six gm. Na-EDTA infused in 4 hours.

† Four gm. Na-EDTA infused in 15 min.

‡ Values at 15 min., 1 hour, 4 hours, 8 hours, and 24 hours following the rapid infusion of 4 gm. Na-EDTA, respectively.

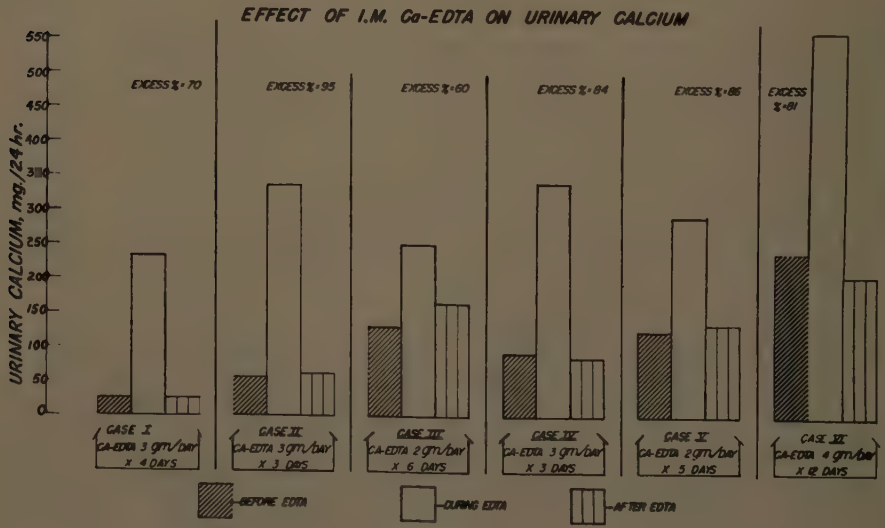

FIGURE 3.

shown that these chelates are poorly absorbed from the gastrointestinal tract. TABLE 8 shows some of the results. The urinary and stool calcium excretions are listed as averages for six-day periods of the study phases before, during, and after the oral administration of Ca-EDTA. The urinary calcium excretion was in approximately the same range during the phase of Ca-EDTA administration as in the control phase, except in patients 1 and 4, who had an

increase of urinary calcium excretion. However, this excess corresponded only to approximately 10 per cent of the theoretically expected excretion. Most of the calcium contained in orally administered Ca-EDTA could be accounted for by an increase in stool calcium.

Another study carried out in a patient who had received a tracer dose of Ca^{45} several weeks before the oral administration of Ca-EDTA had shown that

Table 8
Effect of Oral Ca-EDTA on Calcium Metabolism*

Patient	Before	During	After
	Urinary calcium, mg./day		
1	105	150	170
2	114	125	127
3	54	66	39
4	71	142	79
	Stool calcium, mg./day		
1	254	634	137
2	126	611	283
3	162	658	211
4	157	571	197

* Six gm. Ca-EDTA/day for 6 days. Values are averages of 6-day pools.

Table 9
Effect of Orally Administered Na-EDTA* on Calcium Metabolism

Patient	Before	During	After
	Urinary calcium, mg./24 hours		
1	40	24	22
2	27	10	67
3	53	31	62
	Stool calcium, mg./24 hours		
1	358	129	119
2	122	222	116
3	202	292	114

* Six gm./day given for 6 days. Figures are averages of 6-day metabolic periods.

most of the calcium chelate remained unabsorbed in the gastrointestinal tract. The stool specific activity decreased from 0.26 to 0.07, approximately one-fourth, during the phase of oral Ca-EDTA administration, due to the presence of unabsorbed calcium contained in the calcium chelate.

Table 9 shows the metabolic effects of orally administered Na-EDTA given on six consecutive days. The urinary calcium excretion did not increase during the Na-EDTA phase, which indicates that EDTA had not been absorbed. The stool calcium did not increase, which indicates that calcium had not been

attracted from the body stores for binding with EDTA. Whether the amount of calcium contained in the stool was partially or entirely bound by EDTA in the gastrointestinal tract is not certain.

These studies indicate that Na-EDTA or Ca-EDTA administered orally are either not absorbed, or only partially absorbed, from the gastrointestinal tract and are, therefore, not useful for detoxification or removal of metals that are already deposited in the body.

The determination of chelated calcium is tedious and difficult, and the results obtained may not be reliable. An attempt was made to calculate the amount of ionized calcium excreted on the day of the Na-EDTA infusion. This was done on the basis of the ratio of urinary Sr^{85}/calcium excretion of patients who

TABLE 10*
EFFECT OF NA-EDTA ON URINARY Sr^{85} AND CALCIUM EXCRETION

Patient No.	Type of study	Urinary Sr^{85}, % dose		Urinary calcium, mg./day	
		Day 1	Day 2	Day 1	Day 2
1	Control	8.5	5.6	58	65
	Na-EDTA	3.9	2.8	383 (27)†	346 (33)†
2	Control	21.4	10.4	202	172
	Na-EDTA	9.4	8.1	469 (89)†	463 (134)†
3	Control	7.8	3.6	71	45
	Na-EDTA	2.7	1.6	318 (24)†	341 (20)†

* Courtesy of Spencer *et al.*[17]

† Mg. ionized calcium estimated by Sr^{85}/Ca ratios according to the formula: Ca^{++} exp. = (% Sr^{85} exp./% Sr^{85} control) × Ca^{++} control.

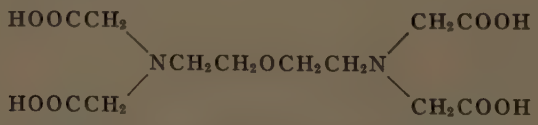

FIGURE 4. Diaminoethylethertetraacetic acid.

have received a tracer dose of Sr^{85} intravenously in the control and Na-EDTA phase. TABLE 10 shows that the excretion of ionized calcium is considerably diminished on the day of Na-EDTA infusion in each of the three patients studied as compared to the preinfusion excretion. This inhibition may well be due to increased tubular reabsorption of calcium in an attempt to maintain calcium homeostasis and to maintain the serum calcium normal.[17]

The excretion, tolerance, and toxicity of several newer synthetic chelating agents and their effect on calcium metabolism in man were recently studied in this laboratory. The formula of one of these agents, BAETA, is shown in FIGURE 4. Its stability constant to calcium is log $K_1 = 10.6$; therefore changes of calcium metabolism induced by this chelate are expected to be similar to that of EDTA. Of interest is the comparative ratio of the stability constant of this particular chelating agent to calcium and strontium as compared to that of EDTA: the affinity of BAETA is higher for strontium than of EDTA,

log K_{Sr} = 9.4 for BAETA as compared to log K_{Sr} = 8.6 for EDTA. Kroll and Siegel,[18] Catsch,[19] and Schubert[20] have recently reported on the effect of this chelating agent on radiostrontium excretion in experimental animals, and Schubert and his co-workers[21] also have reported on the effect of BAETA on plutonium excretion in rats.[21]

Table 11 shows the effect of a 4-hour infusion of 4 gm. Ca-BAETA and of 4 gm. Na-BAETA on the urinary calcium excretion of patients who received a constant, controlled, low-calcium diet. The calciuria increased markedly on the day of the infusion of the sodium and calcium salt of BAETA. The excess calcium excretion was 76 per cent and 92 per cent in the 2 patients respectively when the calcium salt of this chelate was infused; when the sodium salt was administered to patient 1, 57 per cent of the theoretically expected calcium excretion was recovered, but this excess was low in patient 2 (29 per

Table 11
Effect of Na-BAETA* and Ca-BAETA* on Urinary Calcium Excretion in Man

Patient	Chelate	Urinary calcium, mg./day			Excess calcium excretion (%)
		Before	During*	After	
1	Na-BAETA	42	315	19	57
	Ca-BAETA	36	397	26	76
2	Na-BAETA	217	354	213	29
	Ca-BAETA	206	643	263	92

* Four gm. were given intravenously over 4 hours.

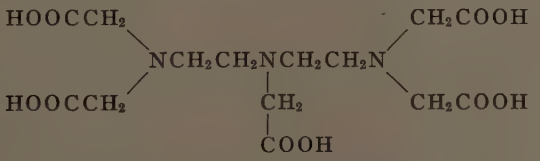

Figure 5. Diethylenetriaminepentaacetic acid.

cent), possibly due to a severe, transient decrease of the blood pressure during the chelate infusion.

Figure 5 shows the formula of another chelating agent, diethylenetriaminepentaacetic acid (DTPA). Its effectiveness on the removal of yttrium from man has been recently demonstrated in our laboratory,[5] and these results are in agreement with those obtained by Foreman[22] and Catsch.[23] The stability constant of Y-DTPA is 20.4 as compared to 18.0 for Y-EDTA.

The effect of Ca-EDTA on the excretion of rare earths was studied at this institution several years ago. Although the stability constant of La-EDTA is rather high (log K = 15.7) the excretion of tracer doses of La^{140}-EDTA in man was found to be surprisingly low, and approximately 5 per cent of the administered dose was excreted via the kidney in 24 hours. Figure 6 shows that the La^{140} excretion could be raised approximately one hundredfold by Ca-EDTA at a time when the La^{140} excretion was very low and most of this rare earth metal was deposited in tissue. The removal of La^{140} from the body

was more effective by the first than by the second administration of Ca-EDTA given in 5 days following the injection of La^{140} as the tracer.[4]

TABLE 12 illustrates examples of the results obtained with DTPA in comparison with EDTA on the removal of La^{140} from man. The urinary excretion following the injection of lanthanum as the chloride served as a baseline excre-

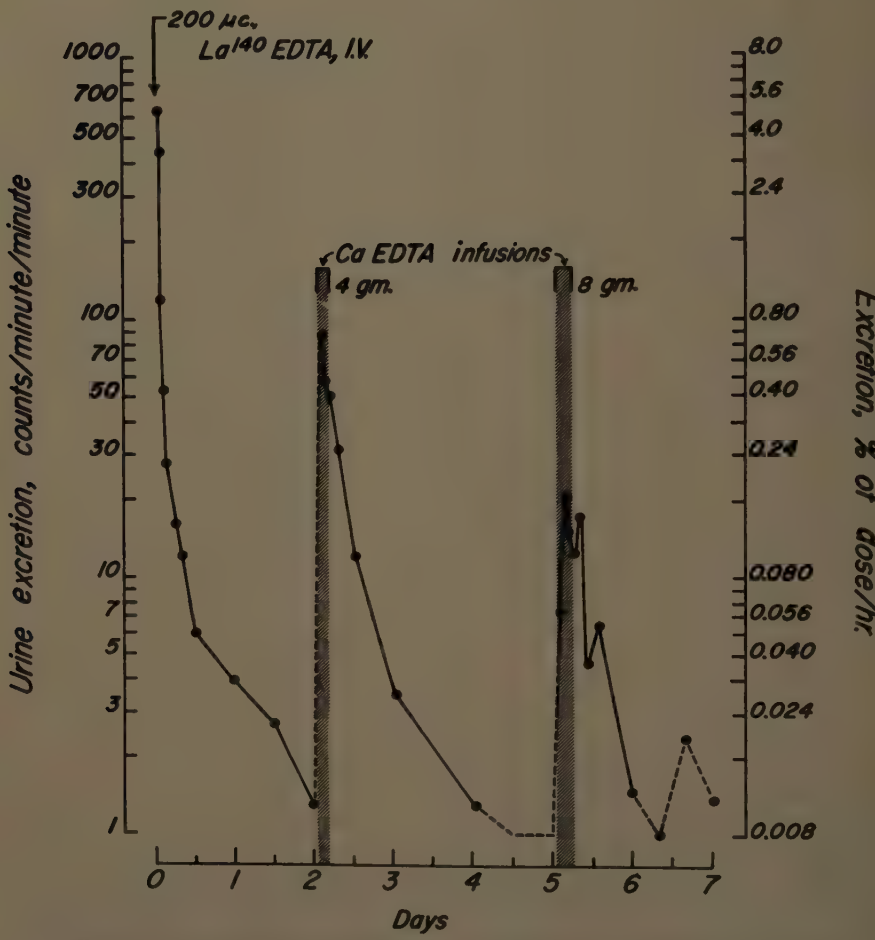

FIGURE 6. (Reproduced by permission of H. Hart and D. Laszlo, Science. **118**.)

tion. Two infusions of 500 mg. EDTA and of equimolar amounts of DTPA were given on 2 successive days starting 24 hours after the injection of $La^{140}Cl_3$. DTPA was shown to be more effective than EDTA in the removal of La^{140} while EDTA led to only a slight or moderate increase of the La^{140} excretion. DTPA raised the excretion from less than 1 per cent to approximately 9 to 12 per cent of the administered dose.

TABLE 13 shows the comparative effect of DTPA and EDTA on the excretion of yttrium in man. Dose levels of 500 mg., 2000 mg. EDTA, and equi-

molar amounts of DTPA were used. The results were similar to those obtained in studies on the removal of La^{140}, and DTPA was more effective than EDTA at either dose level: an approximate tenfold increase of Y^{90} excretion was achieved with 4 infusions of 500 mg. of DTPA each, while only one half the amount was removed with equimolar amounts of EDTA. A fourfold increase of the doses of DTPA did not raise the Y^{90} excretion more than the dose of 500 mg. As little as 100 mg. DTPA, an amount that is very well tolerated, was also quite effective in raising the Y^{90} excretion. Of the 3 dose levels tested, 500 mg. DTPA was considered to be the safest, smallest, and most effective dose.

TABLE 12
EFFECT OF CA-EDTA AND OF CA-DTPA ON La^{140} EXCRETION IN MAN

Patient	Four-day cumulative urinary excretion of La^{140} in % dose remaining 24 hours following La^{140} injection		
	Control	EDTA, 500 mg.*	DTPA, 500 mg.*
1	0.8	2.0	9.6
2	0.9	2.2	12.0

* Given on 2 successive days.

TABLE 13
EFFECT OF CA-EDTA AND OF CA-DTPA ON Y^{90} REMOVAL IN MAN

Patient	Four-day cumulative urinary excretion of Y^{90} in % dose remaining 24 hours following Y^{90}-NTA injection				
	Control (Y-NTA)	Ca-EDTA		Ca-DTPA	
		500 mg.*	2000 mg.*	586 mg.*	2340 mg.*
1	5.0	35.8	42.2	45.1	43.5
2	0.54	23.5	22.1	38.3	32.9

* Given on 4 successive days.

In the course of an investigative program on the metabolism of radioactive trace metals in neoplasia, studies are being carried out with radioactive zinc in man in this laboratory. Tracer doses of Zn^{65} have been injected intravenously, and the excretion in urine and stool have been followed for a prolonged period of time. It was also found that the excretion of Zn^{65} via the gastrointestinal route is as great or even greater than via the kidney.

The stability constant of Zn-DTPA is very high, log $K_1 = \sim 18$, as compared to that of Zn-EDTA, log $K_1 = 12.3$. Therefore the removal of zinc by means of the 2 chelating agents has been investigated. Amounts of 2 gm. Ca-DTPA and of 2 gm. Ca-EDTA were given intravenously at different time intervals following the injection of the tracer. An approximate thirtyfold increase of the urinary Zn^{65} excretion was noted when DTPA was injected on the eighth day following the administration of the Zn^{65} and a fifteenfold increase, respectively,

was achieved when repeated courses of DTPA were given at later time intervals to patient 1. In patient 2 the removal of Zn^{65} was studied as late as 50 days following the intravenous injections of the tracer. A tenfold increase of the urinary Zn^{65} excretion occurred when 4 gm. Ca-EDTA were given intravenously and a similar excess excretion was obtained when one half the amount of DTPA was given. Three infusions of ionic calcium given as the gluconate in amounts equivalent to the calcium content of Ca-EDTA or Ca-DTPA were ineffective in raising the Zn^{65} excretion.

In conclusion, the effectiveness of chelating agents were studied under controlled conditions on various phases of mineral and trace metal metabolism in man. Certain aspects of the regulation of calcium homeostasis in normals and in persons with conditions of derangement of calcium metabolism were clarified. The effect of newer chelating agents with high stability constants for calcium and rare earth metals has been investigated in experimental animals and in man. Further studies of the effect of chelating agents in man should be encouraged in order to study trace metal metabolism in physiologic and pathologic states.

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SYNTHETIC AMINO ACID CHELATING AGENTS AND IRON METABOLISM*

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The structure of the iron atom, characterized by the incomplete filling of the outer orbital electron shells, provides the physical basis for its pronounced tendency for coordinate covalent bond formation. The three dimensional hexacoordinate structural ability of the iron atom serves as a framework for the assembly of electron donor atoms around the iron atom, and has foreshadowed the evolutionary development of iron-containing molecules for their presently known biological functions of catalysis and oxygen transport. One must anticipate that efforts to correct and control the function and flow of iron *in vivo* will succeed only to the extent that it is possible to move nimbly and surefootedly in and around the many aspects characterizing the metabolism of iron *in vivo*. Synthetic chelating agents afford an opportunity, by the inherent variability of molecular structure on their iron-binding ability and metabolism *in vivo*, to modify experimentally the course of the metal in the organism. The present report deals with our efforts to explore these possibilities.

Present concepts of iron metabolism in animals assign to the iron-binding protein of the plasma, transferrin or siderophilin, the specific role of the transport of iron from areas of absorption to those of storage and utilization in the organism.[1] Under normal circumstances the iron-binding capacity of the plasma is only partially saturated, thus affording a measure of iron-binding protection for the animal to allow for possible surges or overloads of iron in the plasma. It is known that signs of acute iron toxicity may be manifest when the plasma iron-binding capacity is exceeded.[2] For this reason, the binding of iron by the transport protein becomes a key value, since it establishes the order of binding strength *in vivo* at which metabolic iron exchange can take place. In a study designed to determine the relative iron-binding of siderophilin and synthetic chelates in plasma, a series of chelating agents of a graded order of iron-binding strength were tested for their ability to remove iron from the iron-siderophilin combination in plasma at neutral *p*H *in vitro*.[3] None of the synthetic compounds were able to effect iron removal from the transport protein under these conditions. On the other hand, when conditions were reversed and study was made of the ability of the transport protein to remove iron in the plasma environment from the preformed iron chelates, it was evident that while the weaker iron-binding chelates of the glycine acetic acid series lost their iron to the transport protein, the more potent iron-binding compounds related to EDTA retained the metal for determinable periods of up to twelve hours. The combination of the two types of experiments suggests that the iron-binding ability of the

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stronger of the synthetic compounds is in the same range of that of the transport protein, and that iron exchange between the two species in plasma is a slow reaction. In order to obtain a more precise measure of the relative iron-binding abilities of DTPA and HOEDTA compared to siderophilin in plasma, the experimental conditions were modified in that the iron was added to the chelate and protein in plasma and the distribution of the metal ion between the two binding agents was determined at various mole ratios of the competing iron-binding materials. If one examines the results (TABLE 1) it is evident that it requires a lower mole ratio of HOEDTA to iron than EDTA to achieve equal distribution of the added iron between the chelates and the protein. In summary, the data from these studies suggest that some synthetic chelating agents have the same order of binding strength as the iron transport protein, and that while exchange of iron between these two types of iron-binding agents in plasma may not be expected, iron available at tissue surfaces or other areas may be distributed between the natural and

TABLE 1
DISTRIBUTION OF IRON ADDED TO SIDEROPHILIN AND CHELATES IN RABBIT PLASMA

Mole ratio EDTA/siderophilin	Percentage iron chelate bound
5/1	20
20/1	50
30/1	75
Mole ratio HOEDTA/siderophilin	
1/1	20
5/1	50
20/1	80
30/1	85

synthetic binding agents in proportion to their respective iron-binding strengths and concentration. The study further warns that chelate iron-binding ability as measured in the rigorously controlled and deliberately simplified systems of the physical chemical laboratory will be only one of the factors that may be determinant in the behavior of synthetic iron-binding chelates in the organism. The ability of the intact organism to metabolize or excrete a specific chelating agent, the kinetics of the compound's reaction with iron, and iron exchange with other binding agents may be more important factors in its effect on iron metabolism than the absolute value for its combination with iron. It may be noted that the variable effects of chelate structure on the kinetics of iron-binding and exchange have been reported.[4] As in our studies, it has been shown that migration of iron from its EDTA chelate to other iron-binding compounds may be a slow reaction whose speed is dependent on the *p*H and other environmental factors.

If we may conclude from the studies described above that iron, in combination with the stronger of the synthetic chelating agents, will not surrender the metal to the plasma iron transport protein nor to the other presumably less potent and less specific iron binding depots of the organism,

what will be the metabolic fate of an injected iron chelate of this type? From the work of Foreman *et al.* with radioactive carbon-tagged EDTA in rats and humans,[5,6] from several other reported studies in other species and microorganisms[7,8] for the same compound, and from our own studies of related amino acid chelating agents[9,10] we know that the amino acid component of these iron chelates is not metabolized and appears essentially quantitatively in the urine within a few hours after parenteral injection. Thus if the metal does not dissociate from the carrier during this metabolic pattern, we may expect that the iron of the more tightly bound amino acid iron chelates will be found in the urine following parenteral administration.

In a series of studies designed to test this possibility, iron chelates were injected by various routes into rabbits and rats, and the urinary and fecal excretion of the metal determined.[11,12,13] The summarized results following intravenous administration (TABLE 2) show an interesting correlation with the *in vitro* studies reported above. Thus the relatively weakly-bound iron of the aminoacetic acid chelate series, which had been previously demon-

TABLE 2
IRON EXCRETION AFTER INTRAVENOUS IRON CHELATES

Compound	Species	Percentage iron dosage excreted in urine
Dibetahydroxyethylimino-acetic acid	Rabbit	8.9
Iminotriacetic acid	Rabbit	7.5
EDTA	Rabbit	66.9
EDTA	Rat	58.6
HOEDTA	Rabbit	69.4
DTPA	Rat	92.5

strated to surrender its iron to the transport protein *in vitro*, was also unable to hold the metal *in vivo* and transport it through the excretory channels into the urine. In contrast, the tightly bound iron of the EDTA and HOEDTA series carried almost 70 per cent of the intravenously injected iron into the urine in a few hours. Of interest is the fact that the more potent iron-binding characteristics of diethylenetriaminepentaacetic acid (DTPA), compared to EDTA and HOEDTA, is correlated with the nearly quantitative excretion of its tagged carrier iron in the urine. Tissue distribution studies of intravenously injected radioactive iron-59 DTPA demonstrate the low level of iron incorporation into hemoglobin and the minimal extent of the tissue deposition of the metal in these normal animals. The essentially quantitative elimination of the tracer iron in the urine also provides conclusive evidence that exchange of the radioactive species of the injected iron compound has not taken place with the iron stores of the body. These results are rather striking when viewed against the generally accepted concept that injected iron cannot be eliminated. The essentially quantitative urinary iron excretion following iron DTPA administration to normal rats and the high level of urinary iron excretion following intravenous administration of EDTA and HOEDTA to rabbits show a noteworthy increase when compared to the maxi-

mum of 27 per cent iron excretion reported by Nissim[14] for the ferric hydroxide-ferrous ascorbate combination.

The metabolic tracer studies of Foreman *et al.* (loc. cit.) showed that, except for oral intubation, the route of EDTA administration had little effect on the metabolism of the compound. A change from intravenous to intraperitoneal, intramuscular, or subcutaneous injection did not modify the high urinary excretion level of the EDTA molecule. Analogously, the excretion of the iron transported by the carrier is also not appreciably changed by the route of administration. As for intravenous administration, intraperitoneal or intramuscular injection of iron EDTA or iron HOEDTA in rats and rabbits results in a 50 to 70 per cent excretion of the metal in the urine within 24 hours. These results are cited for dosage levels of 1.5 to 6 mg./kg. of iron as chelate injected in normal animals. At lower dosage levels, the pattern of iron metabolism with these chelates is altered. At low dosage of radioactive tracer iron EDTA and HOEDTA, a marked decrease in urinary

Table 3

Iron Excretion after Intraperitoneal Administration of Iron Chelates in Rats

Compound	Percentage dose in urine	Percentage dose in feces
HOEDTA	54.8	4.5
EDTA	55.8	14.3
1,2-Diaminocyclohexanetetraacetic acid	90.1	6.4
2-Hydroxycyclohexylethylenediaminetriacetic acid	60.5	18.5
N,*N*′-(2-hydroxycyclohexyl)ethylenediaminediacetic acid	52.8	28.1
Ethylenediamine-di-(*o*-hydroxyphenylacetic acid)	51.3	47.7

output of the metal takes place. We have not yet clearly established whether this result is simply due to radioactive iron exchange with the inert iron stores of the animal or to selective metabolic removal of small quantities of the metal at low dosage levels.

Iron EDTA and iron HOEDTA are examples of strongly bound iron chelates of a highly hydrophilic structure. As with the parent carrier molecules, it is not surprising that the iron chelates are rapidly diffused *in vivo* through the water spaces of the body, followed by the rapid filtration and urinary excretion of these small molecules. Study of the effect of alteration of the organic chelate carrier on the excretion and distribution of intraperitoneally injected iron chelates has demonstrated that this factor may be highly important in modification of the metabolism of the transported iron. The summarized results (TABLE 3) indicate that the chelates of the aryl and cycloaliphatic structures show an increasing tendency to excretion by way of the gastrointestinal tract after intraperitoneal administration. These results bring to mind the long-known structure distribution relations in the organic iodine-containing compounds. A shift from the hydrophilic to hydrophobic structures results in alteration in excretion from the urinary to the biliary tract,

with the consequent well-known selective utility of the compounds in X-ray contrast visualization.[15]

The thesis that iron-free chelates may be able to compete with the transport protein for iron where available in the organism and that once in the chelated form the iron might be carried through the organism to the urinary excretory system has raised the possibility of the utilization of these agents in the treatment of iron storage disease. In this condition the long-term absorption of iron in excess of the needs of the organism results in the massive deposition of the metal in the tissues. In the absence of an experimental counterpart of the human disease in animals, we directed our first efforts to a study of the effect of injected iron-free chelating agents on the urinary iron excretion pattern in normal animals.[16] The injection of HOEDTA as the calcium salt results in a marked increase in the output of urinary iron in normal rabbits (TABLE 4). These results served as the stimulus for the study of the effect of this agent, as well as of EDTA, on the enhancement of iron excretion in hemachromatosis.[17,18] The clinical results, while sufficient

TABLE 4
ENHANCEMENT OF URINARY IRON IN RABBITS BY CHELATES

Compound	Total urinary iron, gamma									
	Pretreatment day		Treatment day				Postreatment day			
	2	4	2	4	6	8	2	4	6	8
Control	100	104	96	98	100	98	100	100	98	96
HOEDTA	100	96	100	140	240	260	250	140	140	
HOEDTA Ethyl ester	100	120	150	170	180	200	380	320	280	160

to demonstrate a marked increase in urinary iron output, were not deemed to be of practical therapeutic value. In an effort to enhance these results HOEDTA was converted to the ethyl ester. The ethyl ester does not chelate iron *per se*, but may be converted by esterase action in the liver to the free amino acid form of the chelate, which is then able to bind the metal. The expectation that this type of metabolic conversion to the active iron-binding agent in the liver might enhance and extend the iron removal effectiveness of the compounds was realized in experimental animals. The urinary iron excretion following intramuscular injection of the HOEDTA ethyl ester (TABLE 4) was indeed higher and more protracted than after the free amino acid. Clinical trial of the compound, however, has indicated a degree of local tissue irritation that may preclude its medical applicability.[19]

More recently, in collaboration with Charles Rath and John Fahey of this institution,* our animal studies with calcium DTPA have been extended to clinical trial. The urinary iron excretion following infusion of this compound in patients with abnormally high iron stores has shown a gratifying correla-

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tion with results to be anticipated from the animal investigations. A test dosage of the compound in patients with normal or low iron stores, as established by marrow aspiration and smear, results in minimal urinary iron excretion. On the other hand, patients with a varying degree of increased iron stores exhibit enhancement in urinary iron output proportional to the degree of tissue iron deposition. For patients with iron storage disease, the chelate-induced iron excretion may reach significant levels. These studies suggest that chelate administration may serve as a convenient measure of the status of the iron stores of the individual and as a specific chemotherapeutic measure for iron elimination.[20]

The problem of treatment of acute iron poisoning, as from the ingestion of ferrous sulfate tablets by children, is different than that of chronic iron overload in iron storage disease. Acute iron poisoning, aside from the local effects of the metal in the gastrointestinal tract, is characterized by extreme elevations in the plasma iron and by interference with metabolic function, especially in the liver. Acute toxic symptoms have been correlated with the elevation of the plasma iron beyond the range of binding of the unsaturated iron-binding protein of the plasma. Efforts to counteract the toxicity of oral ferrous sulfate by treatment with orally administered chelate have not been successful in our hands.[21] The result of iron chelation is the more ready conversion of ferrous ion to the chelated ferric state. It has been our observation that this over-all process increases rather than decreases the toxicity of ferrous ion. This result, we believe, occurs by conversion of the relatively nontoxic ferrous ion to the more toxic ferric HOEDTA chelate. The increased oral toxicity of iron in this chelated form seems associated with the enhanced absorbability of chelated iron from the grastrointestinal tract.

An alternative approach to the problem of the possible treatment of acute iron poisoning rests on the assumption that chelation and excretion of the systemically absorbed and presumably acutely toxic portion of the injected iron should result in amelioration of the consequences of excessive iron intake. While neither calcium EDTA nor calcium HOEDTA yielded promising results in this connection, the effectiveness of injected calcium DTPA has been of a higher order. In a recent case treated here, the increased serum iron levels in a child following ingestion of sugar-coated ferrous sulfate tablets decreased to normal within a few hours after the infusion of calcium DTPA. The urinary iron output was enhanced and the patient exhibited clear and rapid clinical return to normality. Since, however, nonfatal cases of oral ferrous sulfate poisoning sometimes follow this pattern (with the exception of the enhancement in urinary iron output) one must reserve judgement as to the definitive value of the chelate treatment in this case. The results seem promising enough to justify further clinical study.

As we have shown, iron injected in the normal animal need not be fixed in the organism, but may be excreted in the urine to a degree dependent on the organic carrier of the metal. On the other hand, while we know from many studies that the usual forms of injected nonexcreted iron are more rapidly and fully converted into hemoglobin in the iron-deficient animal than in the normal animal, will the water-soluble chelated forms of iron have a similar metabolic utility in the anemic animal? One index of the availability

of injected iron in the iron-deficient animal is the restoration of the hemoglobin levels. TABLE 5 summarizes the hemoglobin response in iron-deficiency anemia in rats following treatment with various forms of intramuscular iron. It may be noted that the iron EDTA and HOEDTA chelates provide an excellent hemoglobin response in these animals, and that the iron saccharate complex is ineffective in providing utilizable iron for hemoglobin formation.

This study is indicative of several important points. It establishes that the route of administration of injected iron may make a profound difference in the iron availability of the metal as a function of the organic carrier. Iron saccharate, considered a useful intravenous iron source, is inefficient as an iron donor when given by the intramuscular route. The reason for the difference in iron utility for this compound by these two injection routes rests on the fact that the intramuscular injection is followed by almost complete local tissue deposition and slow absorbtion of the metal. In contrast to the present data regarding the iron availability of injected iron chelates,

TABLE 5

HEMOGLOBIN REGENERATION IN IRON-DEFICIENT RATS AFTER INTRAMUSCULAR IRON

Compound	Week							
	0	2	4	6	8	10	12	14
	Hemoglobin level gm./100 ml.							
Control	5	5	4.8	4.6	4.4	4.2	4.0	4.0
EDTA	5.6	6.2	7.5	8.2	8.6	9.2	10.2	10.6
HOEDTA	5	7.5	9.2	9.6	9.8	9.8	10.0	10.0
Saccharate	6.2	6.0	5.8	5.8	5.7*	6.2	7.4	8.4

* Tissue necrosis with resultant iron absorption.

it must be noted that a prior report[22] indicated that iron EDTA was ineffective for hemoglobin regeneration in rats when administered by the intravenous route. In the present work, however, we have shown that when the dosage of the chelated form of the metal has been adjusted to allow for its high degree of urinary excretion, the retained portion of the iron is rapidly and efficiently used for hemoglobin formation.[23]

Study of the fate of orally administered iron chelates is complicated by the variety of the possible subsequent metabolic pathways for the metal. The nutritional siderosis of the Bantu and the inherited characteristics of iron storage disease suggest that gastrointestinal iron absorption may be directly affected by the nature of the iron carrier or by some presently undetermined endogenous metabolic transport factor. Were the chelated iron to be absorbed as such, its subsequent metabolic fate would be the same as that of the injected compound. As has been pointed out previously, one of the most significant aspects of the metabolism of injected iron chelates is the high level of urinary excretion of the metal. It is thus evident that if the orally administered iron chelates are absorbed as such from the gastrointestinal tract, some portion of the absorbed chelated iron should rapidly appear in the uri-

nary excretion. Careful study of fractional collections of urine samples from rats following oral intubation of radioactive iron chelates has demonstrated the presence in the urine of significant quantities of iron (TABLE 6).[24] These results, obtained from urine collections made early enough to preclude any possible fecal contamination, and supported in control studies by negative urinary iron excretion after oral inorganic iron, such as ferric chloride, permit the interpretation that some of the orally administered iron chelates are absorbed unchanged from the gastrointestinal tract.

Although radiocarbon-tagged EDTA administered orally, either as the sodium salt or the sodium calcium chelates, passes through the intestinal tract almost completely unaltered and unabsorbed, the evidence presented here indicates that the iron EDTA chelate may be absorbed as such to a modest extent. This conclusion suggests that the nature of the chelated metal may modify the metabolic pattern of the chelating agent. In an alternate manner, one may say that a metal may serve as a cellular transport agent for a given organic molecule.

A well-studied example of this relationship is exemplified by 8-hydroxy-

TABLE 6
URINARY IRON AFTER ORAL IRON CHELATES (RAT)

Compound	Percentage dose in urine
EDTA	9.9
HOEDTA	7.2
DTPA	6.2
Ethylenediamine-di-(*o*-hydroxyphenylacetic acid)	6.3

quinoline (oxine) and its copper chelate.[25] While the metal-free quinoline does not cross the bacterial membrane, the copper chelate provides cellular permeability for the compound. More directly comparable to our findings concerning the oral absorbability of the iron chelates are the observations reported for lead EDTA. When given by mouth, this compound is absorbed and excreted in the urine rapidly enough to provide for urinary tract X-ray visualization.[26] In this case, the change from the calcium to the lead EDTA chelate results in the absorption of the EDTA molecule.

Orally administered iron chelate, which is not absorbed as such, may proceed through the intestinal canal in unaltered form. Clear evidence for this possibility has been obtained, for example, by visual inspection of the gastrointestinal tract and the feces of rats intubated with iron *N*,*N*-ethylenediamine-di-(*o*-hydroxyphenylacetic acid). This iron chelate is characterized by an intense and highly specific red-violet color. Following oral administration, the iron chelate may be seen in sequential passage down the gastrointestinal tract and finally may be clearly evident in the fecal collections.

In addition to the metabolic path of the undissociated iron chelate following oral administration, one may also anticipate some removal of the metal from the carrier in the course of digestion. Reduction of iron to the ferrous state, the presence of competing ligands or anions, metabolic destruc-

tion of the iron carrier by normal metabolic processes or by the action of intestinal microorganisms, and unfavorable effects of *p*H or salt concentration on chelate stability would all tend to produce some dissociation of the chelated iron species. That this process does occur has been substantiated by the observation of the formation of black, iron sulfide-containing feces in rats following oral administration of iron EDTA. Cleavage of metal from the carrier in the course of the digestive processes would permit the absorption and excretion of iron by the mechanisms and pathways characteristic for oral inorganic iron. The variety of these possibilities complicates our understanding of the sequence of events that may follow oral administration of a given iron chelate. Comparison in normal rats of the total iron recoverable in the gastrointestinal tract and feces, following oral intubation of ferric chloride and various chelated iron preparations, offers little evidence that distinction can be drawn between the over-all absorbability of the various iron species in normal animals.

TABLE 7
IRON ABSORPTION IN ANEMIC RATS
(Oral Dosage of 6 mg./kg.)

Compound	Percentage dose absorbed
Ferric chloride	25.0
EDTA	49.9
Ethylenediamine-di-(*o*-hydroxyphenylacetic acid)	32.3
DTPA	45.3
HOEDTA	47.7

In anemic rats, the situation seems somewhat different. Certain of the chelated iron species exhibit increased gross iron absorbability compared to ferric chloride (TABLE 7). Whether these findings are due to an enhanced absorption of the iron chelate in the anemic animal or to the action of the chelate in making the metal more available to the absorbtive mucosal surface is not yet clear.

The work we have described permits us to conclude that by the application of synthetic chelating agents to problems of iron metabolism it will become increasingly possible to direct and channel the distribution of iron *in vivo* for specific therapeutic objectives.

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IRON METABOLISM IN MAN*

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There have been two principal approaches to the investigation of iron metabolism in man: (1) the *in vivo* administration of radioiron, and (2) the *in vivo* and *in vitro* investigation of the properties of ferritin and transferrin (the iron-binding beta-1 globulin).

(1) Iron is transported between body tissues by beta-1 globulin, which has a molecular weight of 87,000 and carries 2 iron molecules. At a physiological *p*H and bicarbonate concentration, the iron beta-1 globulin is extremely stable. The stability constant has been reported as 10^7 by several investigators.[1-3] Warner and Weber[4] calculated the stability constant of the ferric ion to conalbumin, the iron-binding protein of egg white, to be about 10^{29}. They suggested that the iron was chelated to this protein in complexes that apparently contain 3 phenolic groups and 1 bicarbonate group. Since the iron-binding properties of the plasma beta-1 globulin and conalbumin are similar, Warner[5] postulated that their metal-binding sites may also be similar. Data will be presented in this study that confirm the stability constant of the ferric beta-1 globulin to be of the order of magnitude of 10^{29} rather than that of 10^7.

The plasma iron is 0.1 per cent of the total body iron and reflects the balance between that derived from the gastrointestinal tract absorption, from hemoglobin breakdown and from the body stores, and that iron transported to the bone marrow for synthesis of hemoglobin. A low plasma iron may be found in iron deficiency, chronic diseases, uremia, carcinoma, and infection. Only in iron deficiency are the iron stores depleted; they may be increased in the other conditions. Normally, the beta-1 globulin is only one-third saturated. The unsaturated iron binding capacity is usually increased in patients with depleted iron stores; however, there is an overlap into the normal range.

With radioiron it has been possible to get quantitative data on the amount of iron passing through the plasma.[6] Tracer Fe^{59} is cleared from the plasma at an exponential rate. The rate of disappearance is very rapid in hemolytic and iron deficiency patients and prolonged in patients containing hypoplastic marrow. The plasma iron turnover, which is calculated by dividing the plasma iron by the time taken for the Fe^{59} to reach half concentration,[7] reflects the changes in erythropoiesis. The plasma iron turnover was increased in hyperfunctioning marrow; however, in hypofunctioning marrow, as well as in iron-deficiency anemia, a normal turnover may be obtained. This was difficult to explain, since a decreased turnover would be expected.

Although the rate of Fe^{59} incorporation into red cells was found to be related to erythropoiesis, it was also influenced by the amount of unlabeled iron originating from red-cell destruction and the size of the iron pools of the body. It is also impossible to know the quantity of Fe^{59} that proceeds directly to the marrow as compared to the iron originally taken up by other tissues but later

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released to the marrow. The fact that reticulocytes will take up iron directly in the circulating blood, bypassing the bone marrow completely, further complicates the kinetic studies of Fe^{59} utilization.

(2) Iron is stored in the form of ferritin and hemosiderin, which constitutes 23 per cent of the total body iron. Only hemosiderin is detected by the Prussian-blue staining of marrow. Granick[3] originally postulated that only ferritin iron was utilized for hemoglobin synthesis, and that hemosiderin represented deposits of iron in excess of metabolic requirements. Thus the presence of hemosiderin in a bone-marrow aspiration would imply the presence of adequate amounts of iron, and the Prussian-blue test could be used as a rough estimate of the body stores of iron. Shoden *et al.*,[8] however, found a simultaneous labeling of both ferritin and hemosiderin after administering Fe^{59} and found both to be utilized for hemopoiesis. These findings would suggest the necessity of estimating the total quantity of iron in bone marrow, rather than just the hemosiderin fraction. Kerr[9] did a quantitative estimate of iron (per 100 mg. of protein) and compared them with the Prussian-blue method. His results varied considerably, at times, from those obtained by the staining procedure.

The oral iron tolerance test has been used for evaluating the iron stores; however, it has been found to be unreliable by several investigators.[10] Attempts to use the plasma radioiron clearance as a means of estimating iron stores have also been unsucessful.[7] In addition to the limitations involved in the evaluation of the iron stores of man, there are no procedures available for the estimation of the labile iron of the iron stores, nor is there a simple method for the diagnosis of the anemia of infection or for renal disease.

The approach utilized in these studies for the investigation of the anemia of patients involves the intravenous administration of radioiron containing 0.6 mg. carrier iron chelated with ethylenediamine di-orthohydroxyphenyl acetic acid (EDDHA). The half time of the plasma Fe^{59} removal, the urinary excretion of the injected iron chelate (Fe^{59} and total iron), and the erythrocyte uptake of the retained radioiron were determined. The data thus obtained permits the evaluation of the iron stores, the labile iron of the iron stores and the differentiation of anemia due to iron deficiency, infection, renal disease, and decreased erythropoiesis.[11]

Ferric EDDHA

In 1957, Kroll and his co-workers[12] reported the synthesis of the chelating agent EDDHA and estimated a stability constant of 10^{32} for its ferric chelate. The structure of the chelating agent as well as the ferric chelate is shown in FIGURE 1. EDDHA is the phenolic analogue of EDTA; two of the carboxymethyl groups of the EDTA are replaced by phenolic groups. The ferric chelate has four of the coordinating groups located in a plane, another above the plane and the sixth below it.[13] This solution has a deep red color with a molar extinction coefficient of 4650 at 480 mμ. The relationship of the color intensity of this iron chelate to the iron concentration may be seen in FIGURE 2. It is obvious that Beer's Law is obeyed, since the color intensity varies directly with the ferric concentration. Nearly all chelates demonstrate the competition between the metal ion and hydrogen ions. The greater the stabil-

ity of the metal chelate bond, the greater is the concentration of the hydrogen ion required to displace the metal.[14] FIGURE 3 shows the effect of increasing acidity on the metal chelate bond. At *p*H below 3.6, there is a decrease in the color intensity and, at a *p*H of 1, there is complete dissociation of the chelate with disappearance of its color. Advantage is taken of this characteristic of the

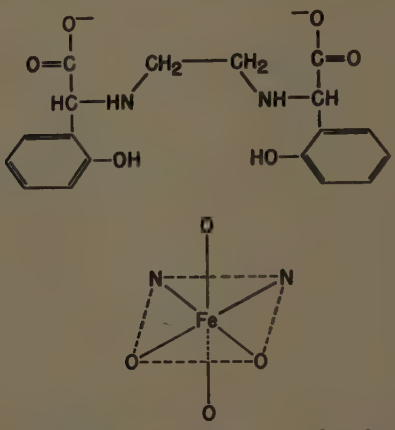

FIGURE 1. Ethylenediamine di(*o*-hydroxyphenylacetic acid).

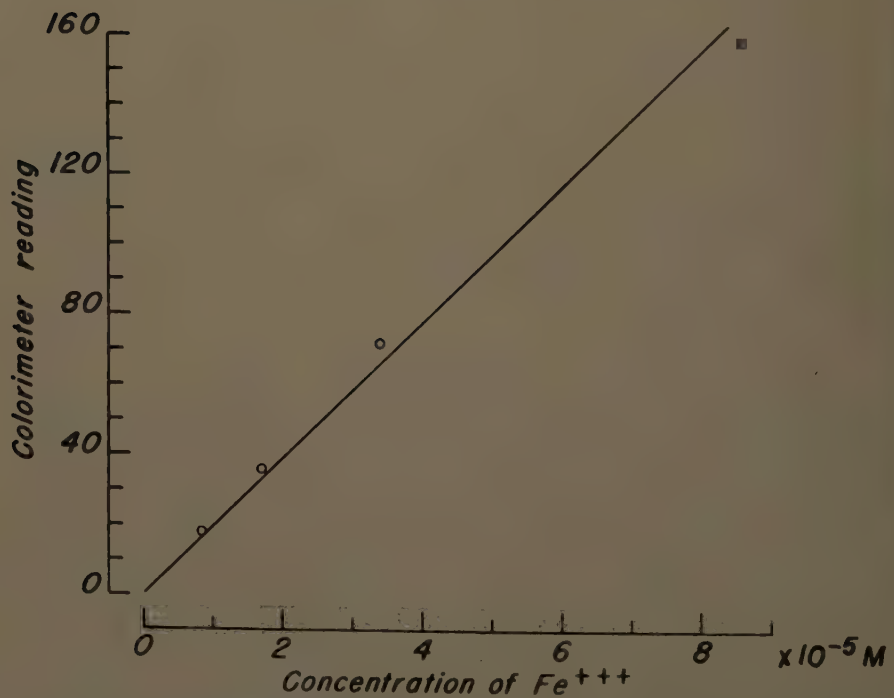

FIGURE 2. Relationship of Fe^{+++} concentration to color intensity. The EDDHA concentration was maintained at 1×10^{-4} *M*.

chelate in determining its concentration in the urine of patients who had the ferric EDDHA administered to them. The change in the color intensity of the urine upon addition of a few drops of concentrated hydrochloric acid permits the calculation of the excreted ferric EDDHA.* The radioiron determination

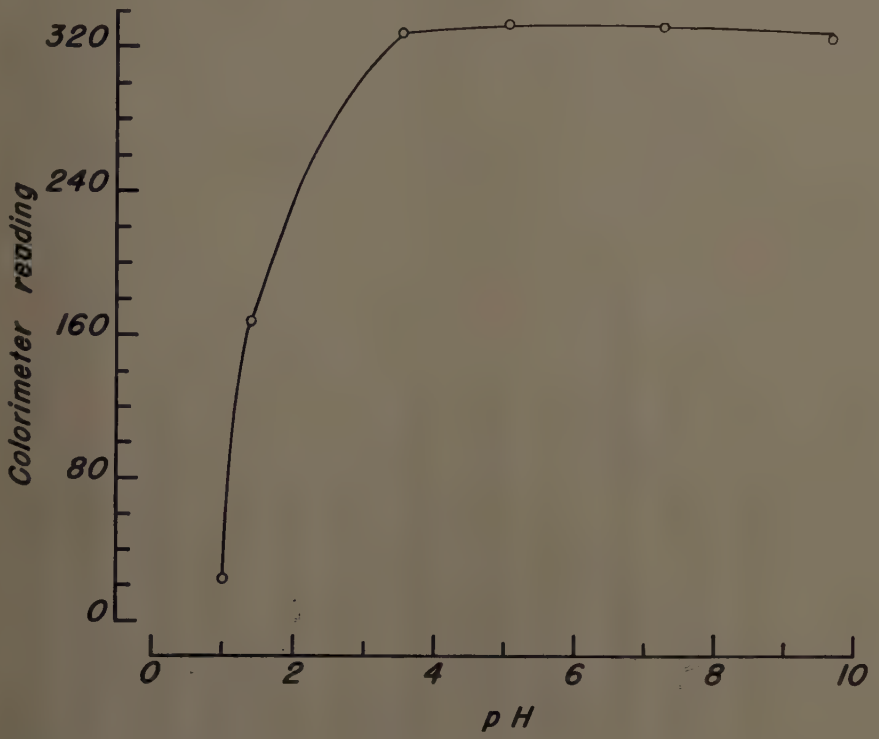

FIGURE 3. Effect of *p*H on color intensity. The Fe-EDDHA concentration was maintained at 1.6×10^{-4} *M*.

on an aliquot of the same urine permits the calculation of the decrease in specific activity of the injected Fe^{59}-EDDHA.

* The Klett-Summerson colorimeter with a 540-mμ filter is used for the colorimetric determinations. Five drops of 6 *N* HCl are added to approximately 10 ml. of centrifuged urine. The acidified urine is immediately stirred with a glass rod, and the decreased colorimeter reading is noted within 45 sec. When the urine remains at this acid *p*H for longer than 1 min., interfering substances may appear. The following medication and diagnostic tests should be withheld for at least 2 to 3 days prior to injection with Fe^{59}-EDDHA, since they interfere with the colorimetric determination for total urinary Fe-EDDHA: streptomycin, dihydrostreptomycin, gantrasin, pyridium, the diagnex test, and the P.S.P. determination.

Another approach to the determination of total Fe-EDDHA excretion is the addition of an excess of solid EDTA to the aliquot of urine. The urine suspension is mixed for 5 min. and then the excess EDTA is removed by centrifugation. The decreased colorimeter reading of the urine is then obtained. Since Fe-EDTA chelate is colorless, the addition of an excess of EDTA to the urine will dissociate the colored Fe-EDDHA to form the Fe-EDTA. The *p*H of the urine falls to only 3.5, and thus there is no need to take an immediate colorimetric reading of the urine, as is required when the HCl is used, since the latter will lower the urine *p*H to below 1. It is only at this very low *p*H that interfering substances may appear in the urine when it stands for more than 1 min.

Administration of Ferric EDDHA to Normal Subjects

With the *in vivo* administration of a radioiron chelate, two conditions may follow. The body may dissociate the chelate and thus only part or none of the administered Fe^{59} is excreted, or the Fe^{59} may exchange with the labile iron of the body's iron stores and the chelate is excreted with a decreased specific activity of Fe^{59}. We found both conditions to occur in the normal subjects.

When the radioiron (containing 0.6 mg. carrier iron) chelated with a 20 per cent molar excess of EDDHA was administered intravenously to hematologically normal patients, 20 to 35 per cent of the administered Fe^{59} was excreted in the urine within 4 hours. The excretion of the administered total iron was greater than that of the Fe^{59}, as seen in FIGURE 4. The difference between

FIGURE 4. Urinary excretion of intravenously administered Fe^{59}-EDDHA in normal subjects.

the total Fe and Fe^{59} excretion represents the exchange of the injected Fe^{59} with the labile iron of the iron stores. The radioiron plasma clearance may be seen in FIGURE 5. It is noted that there is a rapid extracellular distribution of the Fe^{59} in contrast to the intravascular distribution when the Fe^{59} is injected as the citrate or bound to the plasma beta-1 globulin. The half time of plasma radioiron removal was greater than 60 min. in all the normal subjects. Seven to 10 days after administration of the Fe^{59}-EDDHA, a heparinized blood sample was obtained, and the per cent Fe^{59} uptake of the red blood cells was determined. The normal subjects incorporated 45 to 85 per cent of the retained radioiron, as indicated in FIGURE 6.

Administration of the Fe^{59}-EDDHA to Iron-Deficient Patients

The intravenous administration of the Fe^{59}-EDDHA to iron-deficient patients revealed that 20 per cent or less of the administered Fe^{59} was excreted

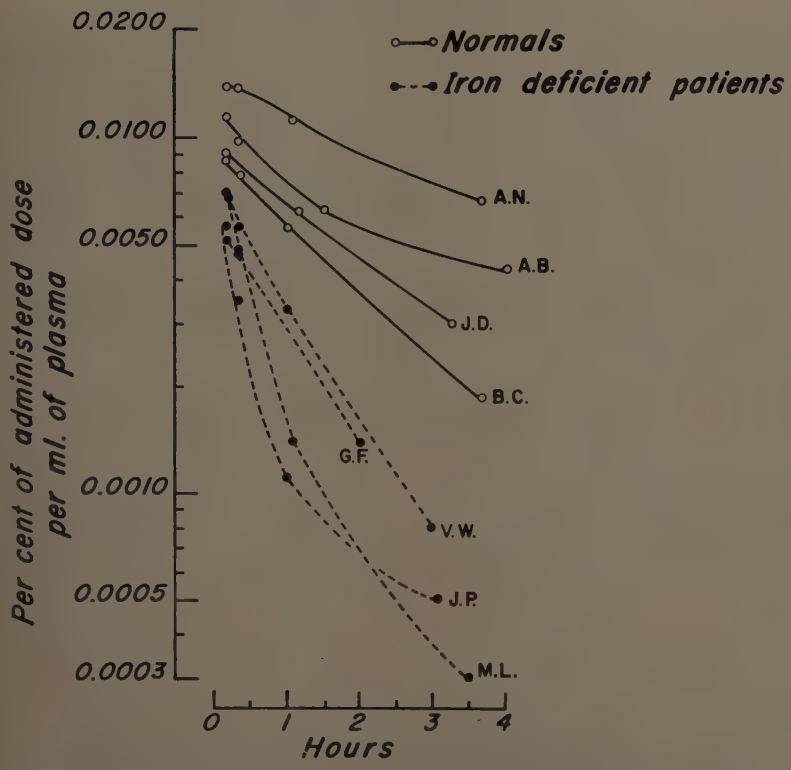

FIGURE 5. Removal of radioiron from the plasma, administered as Fe^{59}-EDDHA.

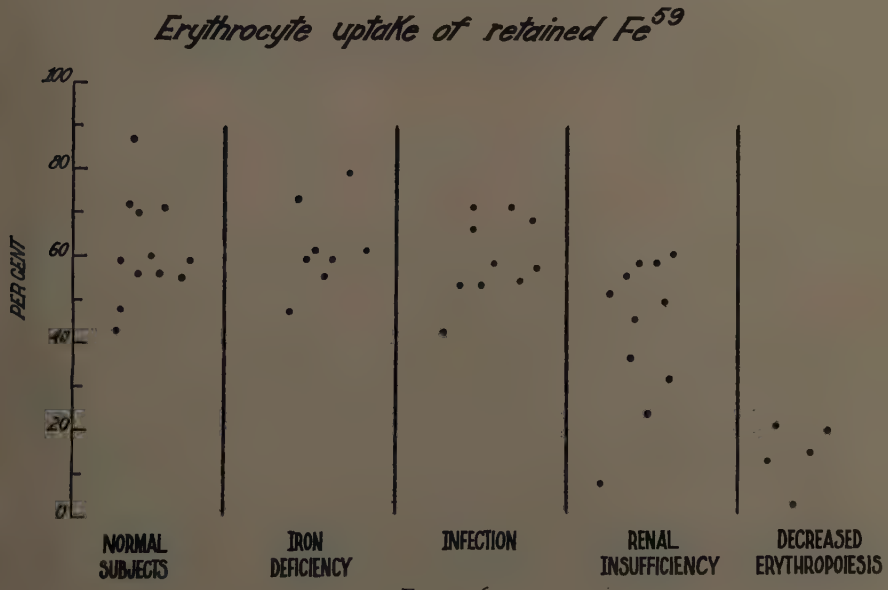

FIGURE 6.

in the urine. As seen in FIGURE 7, in nearly all cases the total Fe excretion was the same as the Fe^{59} excretion. Since the iron stores of the iron-deficient patients are depleted, they have a greater affinity for the chelated Fe^{59}; therefore less is excreted. Due to the decrease or absence of labile iron in the iron stores of these patients, very little or none is available to the Fe^{59}-EDDHA for exchange or binding to the free circulating chelating agent; therefore specific activity of the excreted radioiron is similar to that of the injected radioiron. FIGURE 5 shows the radioiron plasma clearance in these patients. The half time

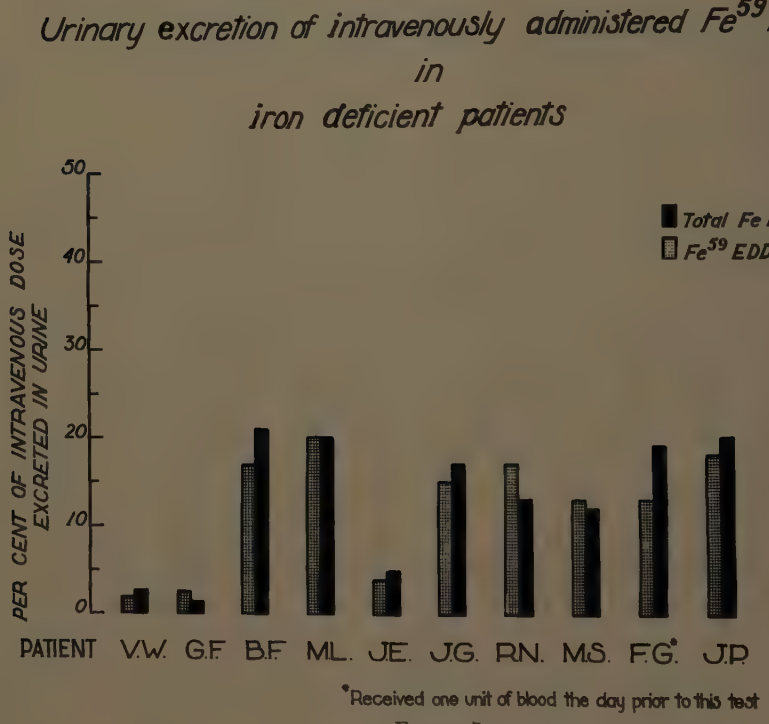

FIGURE 7.

of plasma radioiron removal was less than 60 min. in all cases. The possibility arises that the iron deficient patients have a greater ability to reduce the administered ferric chelate to the ferrous chelate with a subsequent fall in the stability constant of the chelate. This would permit an easier removal of the Fe from the chelate and result in a decreased excretion. The erythrocyte uptake of the retained radioiron for the iron deficient patients was in the normal range as seen in FIGURE 6.

Administration of Fe^{59}-EDDHA to Patients with Chronic Renal Insufficiency

Patients with renal disease excreted less than 20 per cent of the administered Fe^{59}. However, as seen in FIGURE 8, the total Fe excretion was much greater than the Fe^{59}. Since these patients have decreased kidney function,

it is reasonable to expect a decreased Fe^{59} excretion. The fact that the total Fe excretion is greater than the Fe^{59} excretion (resulting in a decrease of the specific activity) indicates that labile iron is present in their iron stores. The radioiron plasma clearance for patients with renal insufficiency was in the normal range. The erythrocyte uptake of the retained Fe^{59} was decreased in one third of these patients, indicating decreased erythropoiesis (FIGURE 6). These findings of decreased erythropoiesis as the fundamental cause of the anemia in patients with chronic renal insufficiency is in agreement with the

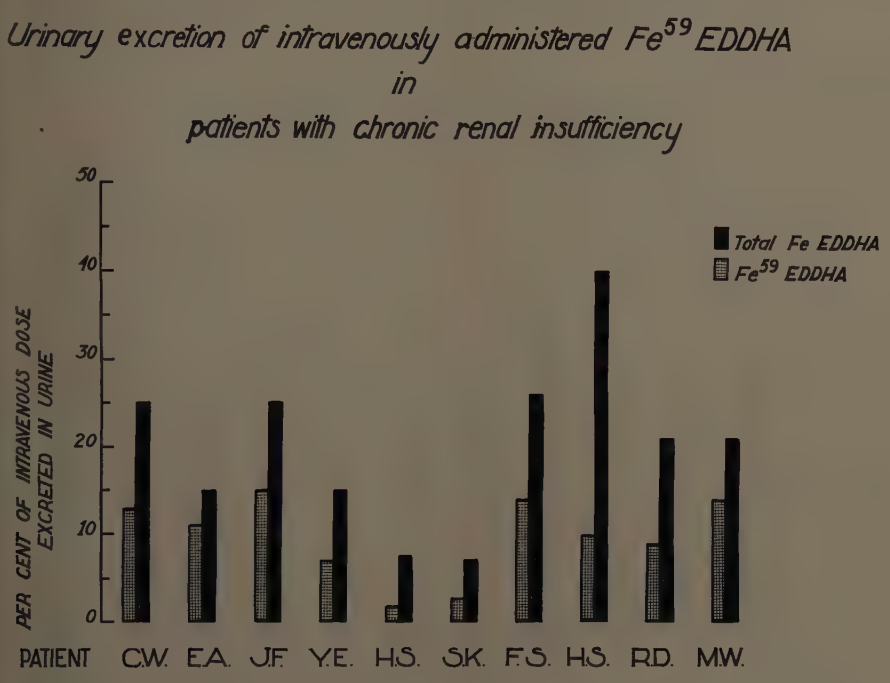

FIGURE 8.

findings of other investigators.[15,16] A hemolytic process may or may not be present.

Administration of Fe^{59}-EDDHA to Patients with Anemia of Infection

The Fe^{59} excretion of patients with anemia of infection was in the normal range, the total iron excretion being very similar to the Fe^{59} excretion, as seen in FIGURE 9. The normal Fe^{59} excretion indicates normal iron stores whereas the slight decrease of the specific activity of the excreted Fe^{59} indicates diminished labile iron. The half time of plasma Fe^{59} removal of patients with anemia of infection varied from 30 to 120 min. Thus the radioiron plasma clearance for these patients fell between the iron deficient and the normal range. The erythrocyte uptake of the retained Fe^{59} was in the normal range (FIGURE 6). The normal erythrocyte uptake indicates that the erythropoiesis

of these patients may be increased if their bone marrow would be presented with a labile form of iron, which in this case is the iron chelate. This observation of the decreased labile iron in patients with infection is in agreement with the findings of Freireich *et al.*[17] that in the presence of an inflammation, there is a delay in the release of iron from senescent red blood cells to the plasma transferrin for new red blood cell production. It also explains why the red cells of patients with infection may have a mild but definite hypochromia,[17-20] a morphological resemblence to the anemia of iron deficiency.

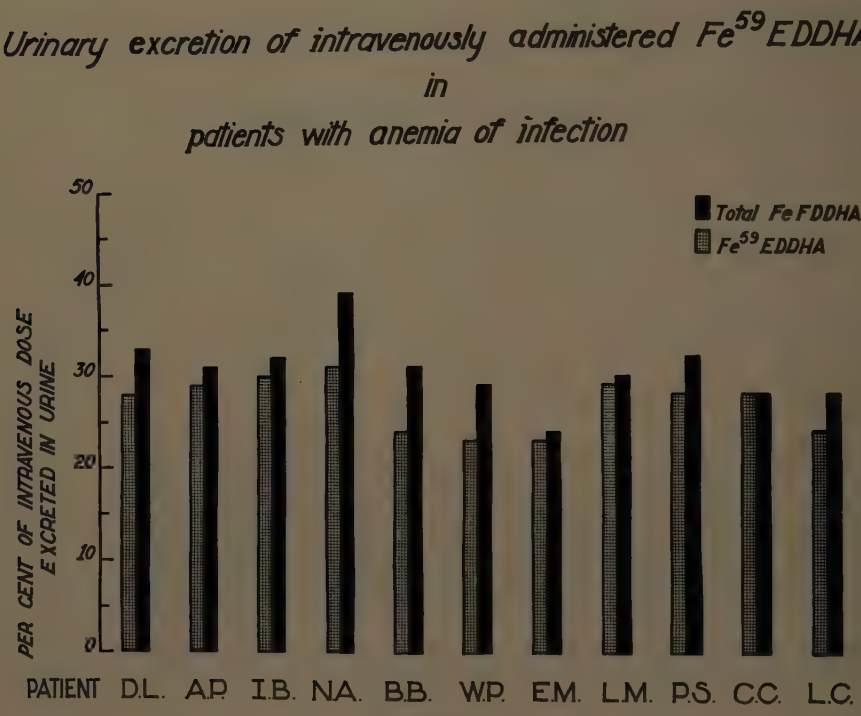

FIGURE 9.

The decreased iron absorption from the gastrointestinal tract during infection, as found by Gubler *et al.*,[21] as well as the failure of iron to be released from the reticuloendothelial cells and senescent blood cells to the plasma at a normal rate could be responsible for the decreased labile iron, despite normal total iron stores. Due to the decreased labile iron, impaired red blood cell production results. Here, too, there may or may not be a shortened survival time of the red cells.[17,22]

Administration of Fe^{59}-EDDHA to Patients with Acute Blood Loss

The Fe^{59} excretion of three patients with acute gastrointestinal bleeding due to peptic ulcers was in the normal range, with the total iron excretion very similar to the Fe^{59} excretion (TABLE 1). The excretion pattern of these

patients, therefore, resembles that of patients with anemia of infection. The normal Fe^{59} excretion indicates normal iron stores, whereas, here too, the slight decrease of the specific activity of the excreted Fe^{59} indicates diminished labile iron. Due to the acute nature of the bleeding, there has been insufficient time to deplete the iron stores, however, the drain on the labile iron is immediate.

In Vitro Incubation of Fe^{59} and Fe^{59}-EDDHA with Normoblasts

It is to be noted that less of the retained Fe^{59} is incorporated into the hemoglobin of red blood cells than when the Fe^{59} is administered as the citrate or

TABLE 1
CLINICAL AND RADIOIRON EXCRETION DATA IN PATIENTS WITH ACUTE GASTROINTESTINAL BLEEDING

Pt.	Age and sex	Hct. (%)	Urinary Fe-EDDHA excretion (per cent of administered dose)	
			Fe^{59}	Total Fe
N.A.	35 M	25	24	30
A.S.	45 F	29	34	34
L.G.	44 F	22	28	31

TABLE 2
IN VITRO RADIOIRON UPTAKE BY NORMOBLASTS OF A PATIENT WITH COOLEY'S ANEMIA

Fe^{59} addition to plasma as	Radioactivity of plasma + normoblasts/radioactivity of plasma	
	Initially	After 4½ hours incubation at 37° C.
Fe^{59}-EDDHA	0.973	0.974
Fe^{59}	0.963	1.018

Plasma concentration of normoblasts was 250,000 per cu. mm. Fe^{59}-EDDHA concentration = 1.1×10^{-5} *M*. Fe^{59} was added to the plasma in tracer amount to assure complete binding by the beta-1 globulin.

complexed with beta-1 globulin. This prompted us to study the radioiron uptake by normoblasts *in vitro*. The blood of a patient with Cooley's anemia with a high normoblast count was obtained. Advantage was taken of the high sedimentation rate of the red cells in separating the normoblasts from them. When these normoblasts were incubated *in vitro* with Fe^{59} beta-1 globulin, the radioiron was incorporated, whereas when the Fe^{59} was chelated to EDDHA, there was no radioiron incorporation by the normoblasts, as seen in TABLE 2. It has been well established that immature red cells can utilize ionic or transferrin iron for hemosynthesis. Jandl *et al.*[23] studied the mechanism of this reaction *in vitro* with human reticulocytes. Since chelated iron is not incorporated by the immature red cells, this bone marrow bypass is eliminated and a better estimation of erythropoiesis is obtained.

The In Vitro Interaction of EDDHA with Crystalline Ferritin

Ferritin consists of the apoferritin protein with a molecular weight of 465,000, and 20 to 23 per cent of the ferritin weight is iron. A small proportion of ferritin iron is capable of dissociating and combining with iron binding agents. This active or labile form may exist as ferric or ferrous iron.

Mazur[24] showed that a small proportion of ferritin iron was capable of dissociating and was thus available as a catalyst for the oxidation of adrenaline *in vitro*. Saltman[25] studied the iron accumulation and efflux from liver slices. He found that there was a passive diffusion of iron through the membrane of

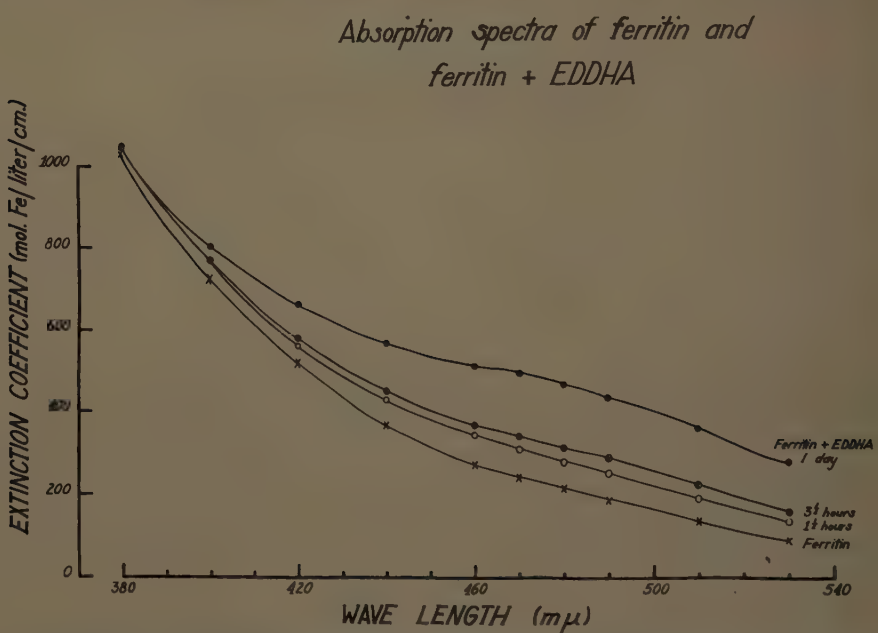

FIGURE 10. The ferritin iron concentration was 4.6×10^{-4} *M*. The addition of EDDHA, 5.5×10^{-5} *M* to the ferritin solution resulted in the absorption spectra at the time intervals noted. The *p*H of the solutions was 7.4 and the temperature was maintained at 28° C.

the cell against an apparent concentration gradient, which did not require energy. We therefore decided to study the availability of ferritin iron to EDDHA. The *in vitro* incubation of crystalline ferritin with EDDHA revealed that 1 to 5 per cent of the ferritin iron was in a labile form and was therefore chelated by EDDHA. The increasing absorption at 480 mμ with time is seen in FIGURE 10. After 1½ hours incubation of the ferritin with EDDHA, 1.3 per cent of the ferritin iron was chelated by the EDDHA. This increased to 2.2 per cent after 3½ hours incubation and to 5 per cent after 24 hours of incubation. This labile iron may be part of the body's labile iron pool that is available for exchange with the injected Fe^{59}. It would therefore follow that in the anemias of iron deficiency and infection there is a decreased availability of this labile iron.

The In Vitro Interaction of Ferric Beta-1 Globulin with EDDHA

In the absence of bicarbonate, the addition of EDDHA (5.5×10^{-5} *M*) to a 90 per cent ferric saturated beta-1 globulin (2.5×10^{-5} *M*) solution gave the absorption spectra seen in FIGURE 11. Fifteen minutes after addition of the EDDHA, 45 per cent of the iron originally bound to the protein was chelated by the EDDHA. After 90 min., 60 per cent was chelated and, after 4 days, 75 per cent could be dissociated from the protein (FIGURE 11).

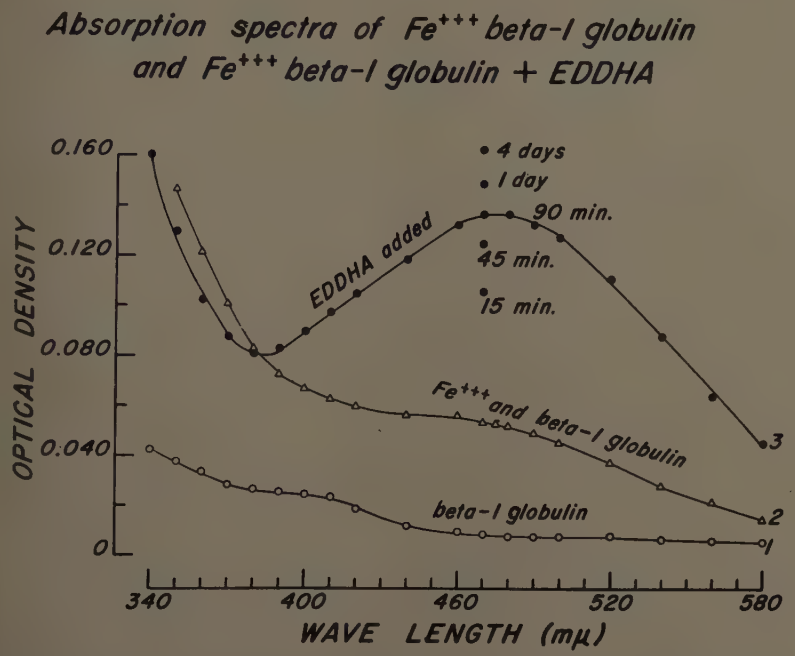

FIGURE 11. The beta-1 globulin concentration was 2.5×10^{-5} *M*. The addition of Fe^{+++} at a concentration of 4.5×10^{-5} *M* to the beta-1 globulin resulted in the absorption spectrum of curve 2. Ninety min. after the addition of EDDHA, 5.5×10^{-5} *M*, to the latter solution resulted in the absorption spectrum of curve 3; the absorption at 460 mμ, at the time intervals after addition of the EDDHA to the beta-1 globulin solution, are designated by the black dots. All solutions were at *p*H 7.4, and ionic strength 0.155.

In the presence of bicarbonate, the extinction coefficient at 460 mμ for ferric beta-1 globulin increases from 1210 to 3400, almost threefold, thus confirming the need of this anion for the complexing of iron to beta-1 globulin. The addition of EDDHA (5×10^{-5} *M*) to 0.02 *M* sodium bicarbonate solutions of one third and two thirds ferric saturated beta-1 globulin (2.5×10^{-5} *M*) revealed that there was no further increase in the absorption maximum at 460 mμ. Thus the chelating agent was unable to dissociate the ferric beta-1 globulin in the presence of bicarbonate.

The reverse phenomenon, the addition of an excess of beta-1 globulin to a bicarbonate solution of ferric EDDHA, did not result in any decrease of the absorption maximum, thus neither was the protein able to dissociate the ferric

chelate. These findings would indicate that the stability constants of the ferric beta-1 globulin and the ferric EDDHA are very close to each other.

The iron exchange between beta-1 globulin and EDDHA was studied by the equilibrium dialysis of Fe^{59} labeled, ferric beta-1 globulin (2.5×10^{-5} *M*) with 4 volumes of ferric EDDHA (1.2×10^{-4} *M*) in a 0.02 *M* sodium bicarbonate solution at *p*H 7.2 for 24 hours at 5° C. When the protein was 33 per cent saturated with iron, there was no exchange between the Fe^{59} complexed to the protein with the iron chelated to EDDHA. With increase of the iron saturation of the protein to 67 per cent, 1 per cent of the Fe^{59} originally bound to the protein was found chelated to EDDHA. The iron saturation of the EDDHA was maintained at 83 per cent.

These *in vitro* studies with ferric beta-1 globulin indicate that this protein iron pool is bypassed with the intravenous administration of the Fe^{59} chelate. The protein does not contribute to the dissociation of the iron chelate nor does it contribute to the exchange of the Fe^{59} for unlabeled iron that results in the decreased specific activity of the excreted Fe^{59} chelate.

Summary

A new technique is described for the evaluation of the iron stores, the labile iron of the iron stores, and the erythropoiesis of patients. This permits, for the first time, the differentiation of anemia due to iron deficiency, infection, renal disease, and decreased erythropoiesis, with one study. Patients with the anemia of infection were noted to have a decrease in labile iron. The defect in the release of labile iron from the iron stores prevents normal erythropoiesis, thus resulting in anemia. Approximately one third of the patients with chronic renal insufficiency were found to have a decreased erythropoiesis as a contributing factor to their anemia.

The *in vitro* interaction of the iron chelate with ferritin, beta-1 globulin, and human normoblasts has been investigated. Incubation of ferritin with EDDHA at 28° C. resulted in the chelation of 2.2 per cent of the ferritin iron by the EDDHA after 3½ hours. In the absence of bicarbonate, the EDDHA was able to dissociate 60 per cent of the iron bound to beta-1 globulin; however, in its presence the chelating agent was unable to dissociate the ferric beta-1 globulin bond. Neither was the beta-1 globulin able to dissociate the ferric EDDHA chelate despite the presence of bicarbonate. Thus the iron binding to beta-1 globulin should be of the same order of magnitude as the ferric EDDHA chelate. Although Fe^{59} bound to plasma beta-1 globulin is incorporated by human normoblasts *in vitro*, the chelation of the Fe^{59} by EDDHA prevents this incorporation.

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UTILIZATION OF SYNTHETIC CHELATES FOR STUDY OF CALCIUM METABOLISM*

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In earlier papers we reviewed studies that provided a basis for the utilization of synthetic amino acid chelating agents in studies of calcium metabolism.[1,2] The present report concerns further study of the use of chelating agents in investigating problems of calcium homeostasis, calcium absorption, and calcium excretion.

Blood calcium levels are maintained by the interplay of mechanisms that control absorption from the intestinal tract, urinary and fecal excretion, movement of calcium in and out of the vascular system, deposition and solution of the mineral from the skeletal system, the vitamin D nutritional status, especially of the young of a species, the functional state of the thyroid and parathyroid and, perhaps, other presently undelineated factors. The addition to blood of a chelating agent such as ethylenediaminetetraacetic acid (EDTA) results in the immediate formation of the calcium EDTA chelate, which is then excreted by the urinary system. In effect, the injection of EDTA results in an abrupt sharp decrease in the calcium blood levels, which forces the organism to institute all possible measures for the restoration and conservation of blood calcium. We have shown previously that the rate and extent of EDTA hypocalcemia may be controlled by means of the route and the rapidity of EDTA administration.[3] These early results have been confirmed and extended in respect to other species.[4-6]

We have studied the extent and duration of hypocalcemia induced by intraperitoneal (I.P.) injection of EDTA in Osborne Mendel rats. For adult animals the maximum tolerated EDTA dose of about 0.0006 moles/kg. results in an abrupt drop of oxalate-precipitable serum calcium levels to 4.5 mg./100 ml. in a 15-min. period. Restoration of the serum calcium to normal levels is a fairly slow process requiring about 24 hours. It is of some interest that the return to normal calcium levels in these adult animals is at least a biphasic process. A plot[7] of the logarithm of serum calcium levels against time yields two straight lines with an intercept at a calcium level of approximately 7 mg./100 ml. It is tempting to consider the possibility that this experiment provides support for the concept advanced by McLean and Urist,[8] that a rapidly acting mechanism involving the release of calcium from bone stores supports a blood calcium level of 7 mg./100 ml., whereas a slower system, perhaps dependent on parathyroid mediation, serves to regulate the slower increase of blood calcium to the normal level of about 10 mg. The response of young rats to hypocalcemic challenge by I.P. EDTA injection differs from the adults. Restoration of the blood calcium levels to normal occurs rapidly and is com-

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plete in the three-hour period in which only the first and more rapid of the adult blood calcium restorative processes is important. Based on the assumption that the bone provides the first and most important, rapidly available source of calcium for maintainence of blood levels, these results suggest that the skeletal system of the young has a more mobile calcium fraction and is more proficient at this homeostatic task than that of the adults. The more vascular nature of young bone and its smaller relative content of dense bone tissue is, of course, in keeping with these concepts. Of further interest is the fact that young animals are better able to withstand the challenge of EDTA-induced hypocalcemia than adults. In our colony an I.P. EDTA dose of 0.008 moles/kg., which is usually fatal to adult rats, is tolerated by young.

As might be anticipated, the response of rachitic rats to hypocalcemic challenge by EDTA injection is greater than that of their normal fellows. At I.P. dosage of the chelate of 0.008 moles/kg. rachitic animals respond with a blood calcium decrease to 3 mg./100 ml. in 15 to 30 min. Restoration to normal levels is essentially complete in a two-hour period. In this respect the rachitic animal behaves identically with his normal peer. The difference in intensity rather than in quality of the response argues that the primary mechanism of physicochemical replacement of blood calcium from bone is identical in the two groups but is slowed by the low level of available mineral in the rachitic animal.

The development of an experimental animal with a reproducible and controlled hypocalcemia and blood calcium restoration has permitted a new approach to certain aspects of the question of the absorbability of oral calcium preparations. An extensive literature deals with the factors involved in control of the absorption of oral calcium. The virtues as well as the problems involved in the methodology of calcium balance studies and radioactive calcium absorption techniques have been summarized and are well recognized.[9] In the acutely hypocalcemic animal described above, all available mechanisms are maximally at work to restore the depressed blood calcium levels. If this animal is provided with a source of absorbable calcium in the intestine, we may reasonably expect that the restoration of blood calcium levels will be speeded by influx of absorbed calcium to blood. To test this process experimentally we have utilized the EDTA-hypocalcemic animal at the low point of the hypocalcemic response 15 min. after the I.P. injection of the chelate. With this animal as a control we have then measured blood calcium changes induced by the intubation of calcium preparations.

Time of Oral Calcium Absorption

A soluble, well-absorbed calcium salt, calcium gluconogluconate, was intubated at various time intervals in relation to the sampling of blood calcium levels at the 15-min. hypocalcemic low point. Calcium solution intubated in the period of 15 to 45 min. prior to the sampling time produced an elevation of control blood calcium to levels between 4.5 to 6 mg./100 ml. The increased blood calcium of the treated group reached a peak point at 45 min., after which it decreased to control values. Calcium solutions intubated before or after this period were without effect on the hypocalcemia. These results support

other evidence that soluble calcium salts are rapidly absorbable and that this absorption occurs at the time calcium is in the proximal portion of the intestinal tract. The work further provides a basis for the examination of the comparative absorbability of calcium preparations. The calcium EDTA chelate, for example, may serve as a model of a tightly bound nondissociated calcium compound. If we test the absorption of calcium after orally administered calcium EDTA by the presently described technique, we can find no rise of blood calcium level in the hypocalcemic animal. These findings are in agreement with previous reports on the poor availability of calcium in ingested calcium EDTA. In contrast to calcium EDTA, other soluble calcium salts such as the lactate and the gluconate produce an elevation of the blood calcium in the hypocalcemic animal.

The method described for testing the absorbability of calcium compounds is a short-term one, and has been less reliable or useful in tests of the availability of slowly absorbed calcium sources. Thus calcium phosphate and calcium phytate suspensions require longer periods of time for movement through the gastrointestinal tract than do the soluble salts, and are not rapidly enough absorbed to modify the experimental course of acute EDTA hypocalcemia. While the kidney excretes calcium in small quantity, even under such conditions of calcium deprivation as starvation, it is clear that the organ must have a highly selective ability to regulate the conservation of the ion. The severely hypocalcemic animal might be expected to invoke available renal mechanisms to conserve the excretion of calcium presented to the tubule. It was of interest to determine whether extreme hypocalcemia would cause retention of calcium from a tightly bound calcium chelate such as calcium EDTA. It has been suggested that the kidney tubule is unable to reabsorb nonionized calcium complexes.[10]

Well-documented studies have established that the injection of calcium EDTA is followed by a renal excretion of between 75 and 100 per cent of the injected calcium.[11,12] These reports, coupled with the evidence that nonionized calcium EDTA does not substitute for the physiological action of ionized calcium salts, have led to the conclusion that the compound is of no value as a parenteral source of calcium.[13,14] On the other hand, some clinical evidence has been brought forward to support the contention that in the calcium-deficient patient the injection of calcium EDTA is followed by retention of calcium and amelioration of the symptoms.[15,16] These seemingly contradictory reports would be resolved if the hypocalcemic state resulted in the initiation of a mechanism able to bring about the retention of calcium from injected calcium EDTA.

In testing this question we have first established that in normal adult rats the I.P. injection of calcium EDTA is followed by excretion in the urine of an average of 75 per cent of the injected calcium in the first 24-hour period. When the animals are rendered hypocalcemic, with blood calcium levels of 4 mg./100 ml. of oxalate-precipitable calcium, the excretion of calcium EDTA is reduced to 30 per cent of the dose. Analogous results previously were reported by us for the excretion of the combination of calcium and magnesium in the urine.[17] Since the physical factors that govern the separation of calcium from

its EDTA carrier are known[18] we have tried to determine whether they might also control this dissociation *in vivo*. The foremost of the factors governing the stability of calcium EDTA is the *p*H of the environment. While the complex is stable on the alkaline side, dissociation is rapid and complete as the *p*H falls to about 5.0. To test whether renal *p*H levels might be involved in the cleavage of calcium from calcium EDTA, we pretreated the experimental animals with Diamox, an agent clinically effective in producing an alkaline urine by inhibition of carbonic anhydrase in the kidney. The inhibition of the enzyme limits the ability of the kidney to secrete hydrogen ion for renal exchange with sodium ion. Hypocalcemic animals treated with Diamox were unable to retain the calcium of injected calcium EDTA. All of the element was excreted in the urine with the carrier.[19] We may conclude that calcium may be reabsorbed in the kidney tubule where the carbonic anhydrase-hydrogen ion secretion mechanism functions. The same sequence of events takes place when alkalinization is brought about by administration of sodium bicarbonate.

It seems possible that the reabsorption of renal calcium from calcium complexes is dependent on the dual factors, *p*H stability of the calcium complex and the regional acidity levels in the kidney. Synthetic chelates with known *p*H calcium-binding characteristics offer a means of exploring these relations. Our first studies in this laboratory exhibit some promise in this connection.[20,21]

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THE INTERACTION OF YTTRIUM CHELATES WITH SERUM CONSTITUENTS*

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Studies carried out with radioactive rare earth isotopes in this laboratory have been motivated by a twofold interest, namely, the decontamination of these by-products of fission and their possible usefulness in the diagnosis and treatment of cancer. Certain rare earths form complexes with nucleic acids[1] and, therefore, it may be possible to achieve selective localization of radioactivity in tumor tissue.[2,3] The tissue distribution of rare-earth metals in experimental animals can be modified by changing the form in which the rare earth is administered; that is, as an ionic salt or metal chelate, and the localization can be controlled by administering the metal in the form of *different* chelates.[4,5] Thus the possibility of directing the radioactive rare earths to the site of the cancerous growth exists.

The experiments reported here are an attempt to investigate the nature of the interaction of chelated yttrium with serum and serum constituents *in vitro*. The techniques of pressure filtration and equilibrium dialysis, which have been previously described, were employed.[6,7] The yttrium chelates used in the studies to be presented are listed in TABLE 1.

An example of the tissue distribution of different rare-earth chelates is illustrated in FIGURE 1, which shows radioautographs of kidney sections of mice injected intravenously with the yttrium chelates Y^{91}-HEIDA and Y^{91}-DHEG. When Y^{91}-HEIDA was used, radioyttrium was found to localize chiefly in the cortex of the kidney; when Y^{91} was administered as the chelate Y-DHEG, the radioactivity was diffusely distributed throughout the organ.

Excretion studies carried out with yttrium chelates have shown that the magnitude of excretion depends on the chemical form of the metal, that is, whether free metal ions are available to interact with body constituents or whether the metal ions are chelated and thus protected from such competitive interactions. If a rare earth is injected as a metal complex that does not appreciably dissociate, the rare earth will be largely excreted in chelated form. If dissociation occurs to any considerable extent the amount of metal that will be removed from the chelate and distributed in the body depends on the competition between the binding power of the body constituents and the injected chelate.[4,5,9]

FIGURE 2 shows the results of equilibrium dialysis experiments on the binding of yttrium chloride with albumin, β'-globulin and γ-globulin, conducted at a temperature of 5° C. and at pH 6, using Y^{91} as the radioactive label. This figure shows that the binding of yttrium to the three serum proteins is similar at concentrations of free yttrium ranging from 1×10^{-4} to 1×10^{-2} M. The greatest binding achieved under these conditions was 9 moles of yttrium per

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mole of protein. It has also been shown in other experiments that these reactions are reversible, and that yttrium is completely diffusible under these conditions in a control system with saline.[7]

The filterability from serum of a series of Y^{91}-labeled yttrium chelates was

TABLE 1
LIST OF YTTRIUM CHELATES

Symbol	Chemical name
Y-DTPA	diethylenetriaminepentaacetate
Y-CDTA	cyclohexane *trans*-1,2-diaminetetraacetate
Y-IPDTA	isopropylenediaminetetraacetate
Y-EDTA	ethylenediaminetetraacetate
Y-BAETA	bis(2-aminoethyl) ether tetraacetate
Y-EDDHA	ethylenediamine di(*o*-hydroxyphenylacetate)
Y-HCDTA	*N*(2-hydroxycyclohexyl) ethylenediaminetriacetate
Y-HEEDTA	*N*(2-hydroxyethyl) ethylenediaminetriacetate
Y-NTA	nitrilotriacetate
Y-HEIDA	*N*(2-hydroxyethyl) iminodiacetate
Y-DHEG	*N*,*N*,di(2-hydroxyethyl) glycine

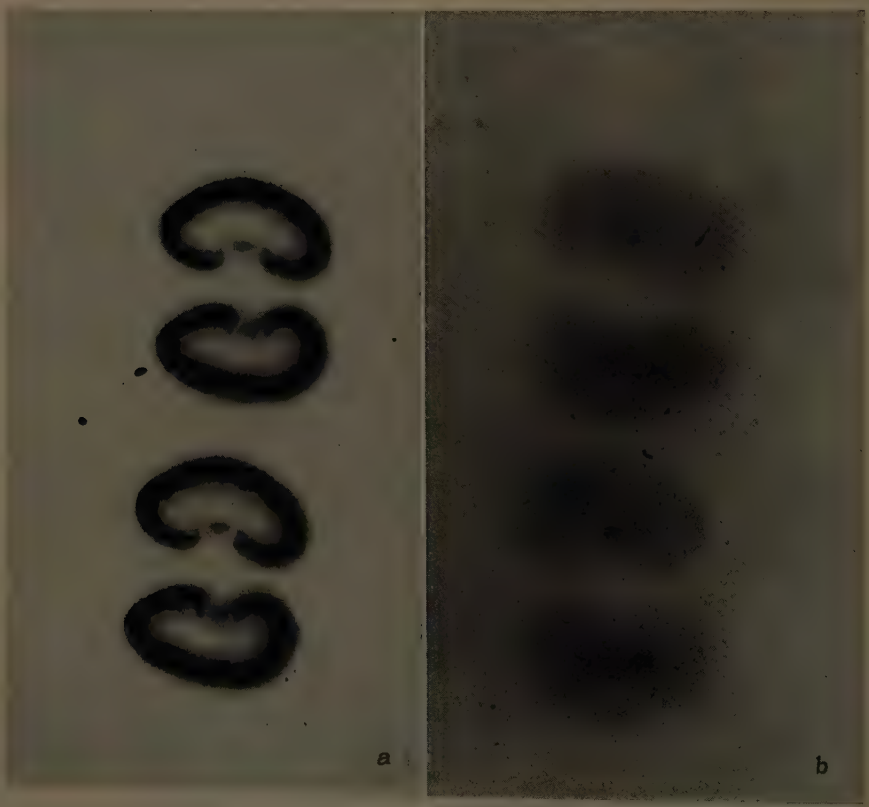

FIGURE 1. Radioautographs of kidney sections of mice injected with yttrium chelates. (*a*) Y-HEIDA. (*b*) Y-DHEG.

determined by radioassay of the per cent of yttrium[91] that diffused through a cellophane membrane during centrifugation.[6,7] The results of these experiments are listed in TABLE 2. The yttrium chelates are arranged in order of decreasing stability constants (log K_1). The concentration of yttrium in this series is 1×10^{-4} M, and the ligand to metal molar ratio is 2:1. In the control studies carried out with saline at a pH of 7.6, the filterability of all the yttrium

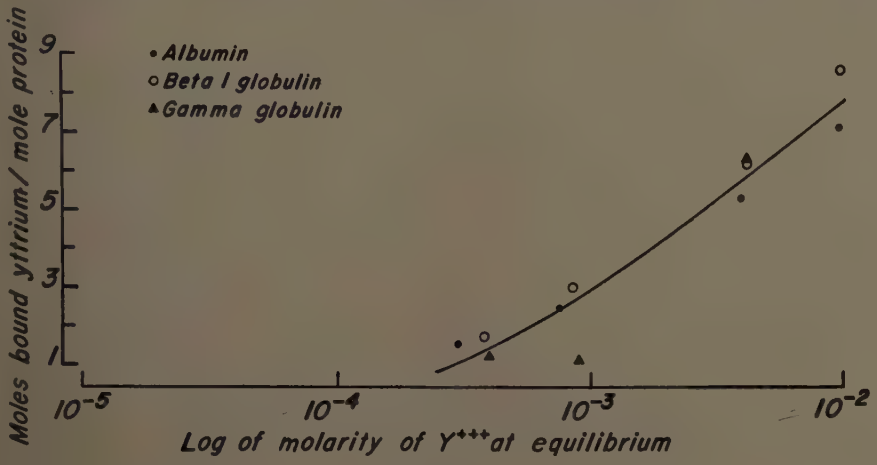

FIGURE 2.

TABLE 2

INTERACTION OF YTTRIUM CHELATES WITH SERUM AND SERUM PROTEINS

Y-Chelate	Log K_1	Per cent filterable yttrium		Moles of yttrium-bound	
		Saline pH 7.6	Serum pH 7.6*	Albumin pH 7.6	Gamma globulin pH 7.6
Y-DTPA	20.3	96	121	No binding	No binding
Y-CDTA	19.15	99	109		
Y-IPDTA	—	103	106		
Y-BAETA	18.50	101	105		
Y-EDTA	18.09	95	106		
Y-EDDHA	—	93	7.1	0.078	
Y-HCDTA	—	97	5.9	No binding	
Y-HEEDTA	14.49	99	2.5		
Y-NTA	11.41	95	0.95		
Y-HEIDA	8.6	88	0.93		
Y-DHEG	—	52	0.23	0.47	

* Human.

chelates was approximately 100 per cent. Yttrium chloride forms hydroxy colloids at this *p*H and has a filterability from saline ranging from 13 per cent to 25 per cent. Yttrium-DHEG, a very weak chelate, seems to behave like yttrium chloride under these conditions. In filtration studies carried out with human serum at *p*H 7.6, a relationship between the stability constants and the filterability of yttrium chelates was observed. Yttrium is approximately 100 per cent filterable from those yttrium chelates with high stability constant (log $K_1 > 18$), for instance, Y-DTPA, Y-CDTA, Y-IPDTA, Y-BAETA, and Y-EDTA. The filterability of more than 100 per cent is probably due to the fact that no correction was made for the lower water content per unit volume of serum than per unit volume of filtrate. The filterability of yttrium chelates of intermediate stability (log K_1) between 14 and 18, (Y-EDDHA, Y-HCDTA, and Y-HEEDTA) ranges from 2 per cent to 7 per cent, suggestive of considerable dissociation of the yttrium chelate complex and subsequent formation of nonfilterable yttrium compounds. Very weak yttrium chelates (log $K_1 < 14$) show less than 1 per cent filterability and yield results similar to yttrium chloride.

Since it was shown that *ionic* yttrium is bound by albumin and γ-globulin it was of interest to study the binding of chelated yttrium to these protein fractions. Such studies may determine whether some of the results obtained in pressure filtration experiments with metal chelates that show a low filterability and therefore dissociate readily are due to yttrium protein binding. In addition, using metal chelates in binding studies at physiological *p*H might facilitate carrying out studies with heavy metals that form hydroxy precipitation at *p*H 7. Gurd and others[8] have suggested this line of investigation. In the experiments reported here, equilibrium dialysis was used. The concentration of yttrium chelates was the same as for the pressure filtration experiments, the molar concentration of albumin was 4.2×10^{-4} *M*, and that of γ-globulin was 0.69×10^{-4} *M*. The last 2 columns of TABLE 2 show that there was essentially no binding of yttrium to the proteins under these conditions except in 2 instances. These results were anticipated in binding studies using strong chelates that have a stability constant of log $K_1 > 18$, in view of the results obtained in pressure filtration studies. Although there is substantial dissociation when weaker yttrium chelates are used, the stability constant or binding strength of yttrium to the chelate appears to be greater than of yttrium to protein and, therefore, protein does not bind yttrium. However, the filterability of Y-DHEG is similar to that of yttrium chloride, and this finding was confirmed by control experiments carried out with saline indicating that all the chelates but Y-DHEG give 100 per cent recovery. Results with Y-EDDHA will be discussed in a subsequent section.

Studies carried out in experimental animals, in man, and in pressure filtration experiments have shown that a substantial amount of yttrium becomes nonfilterable under physiological conditions when chelates with weak or intermediate binding strength were used.[2,6,7] Also, the present studies of the binding of yttrium chelates with protein indicate that the formation of nonfilterable compounds is not due to binding of yttrium with albumin or γ-globulin. It was therefore of interest to study the effects of other components of the serum on the filterability of yttrium chelates.

TABLE 3 presents the results of pressure filtration experiments carried out with the same series of yttrium chelates, using bovine serum and bovine serum filtrate. The data indicates that the diffusible serum components, the serum filtrate, behave similarly to the total serum in removing yttrium from less stable chelates to form nonfilterable aggregates. The diffusible serum components

TABLE 3
FILTERABILITY OF Y^{91}-CHELATES FROM BOVINE SERUM AND SERUM FILTRATE

Y-Chelate	Log K_1	Per cent filterable yttrium from	
		Bovine serum *p*H 8.3	Bovine filtrate *p*H 8.3
Y-DTPA	20.3	113	102
Y-CDTA	19.15	95	97
Y-IPDTA	—	107	88
Y-BAETA	18.50	92	93
Y-EDTA	18.09	100	89
Y-EDDHA	—	29	38
Y-HCDTA	—	16	32
Y-HEEDTA	14.49	3.4	4.7
Y-NTA	11.41	0.3	3.0
Y-HEIDA	8.6	1.0	1.2
Y-DHEG	—	0.4	0.2

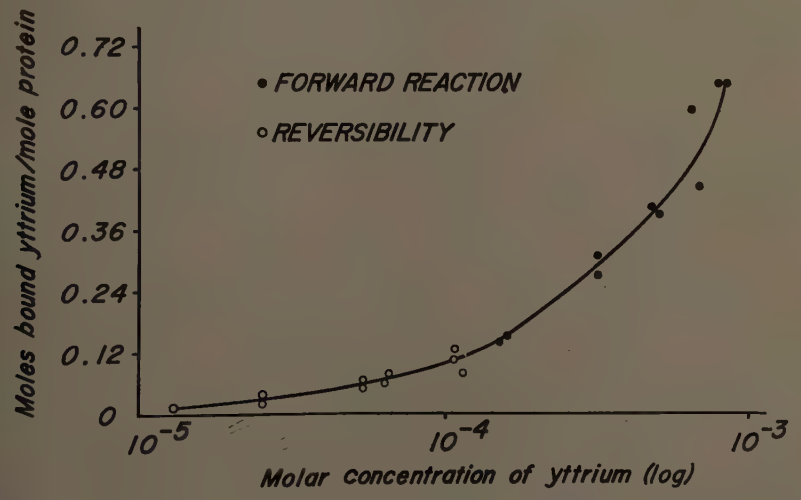

FIGURE 3. Binding of chelated yttrium (Y-EDDHA) to albumin; equilibrium dialysis.

are obviously stronger competitors for yttrium than albumin and γ-globulin. In addition, one must consider that in a complex biological medium, such as blood, the separate components might interact with metals or metal chelates in a different manner than the total serum.

The interaction of Y-EDDHA and albumin was further investigated to determine the binding at different concentrations and to test whether the binding was reversible. FIGURE 3 shows the results of equilibrium dialysis studies

carried out with yttrium-EDDHA in a chelate to metal molar ratio of 2:1. The albumin concentration was maintained constant. When the molar concentration of the yttrium chelate was varied from 10^{-5} to 10^{-3} M, the moles of yttrium bound per mole of albumin increased. Although FIGURE 3 shows the yttrium molar concentration in logarithmic units, when the molar concentration of yttrium is compared directly on an arithmetical scale with the moles of yttrium bound per mole of albumin, a linear relationship can be demonstrated. In order to determine whether the reaction was reversible after the first equilibrium was established at 24 hours, a second equilibrium was established at 48 hours against saline at a lower concentration of free yttrium. The values of the second equilibrium are in good agreement with those anticipated, since they fall on the same curve and thus indicate that the reaction is reversible. A possible explanation as to why an interaction between Y-EDDHA and albumin occurs, and does not occur with the other chelates might be found in the structure of this chelate, which differs from the other chelates in that it contains phenolic groups. The linear relationship between yttrium concentration and yttrium binding suggests the possibility of yttrium-chelate-protein binding. Because of the phenolic groups the chelate may react with the side chains of the albumin, thus forming a bridge between yttrium and albumin. Since EDDHA has an absorption spectrum in the ultraviolet, it was possible to determine spectrophotometrically the concentration of EDDHA at equilibrium. Preliminary results indicate that the EDDHA concentration parallels the yttrium concentration; therefore the assumption of yttrium-chelate-protein binding may be correct. This line of investigation will be further pursued.

Summary

The nature of the interaction of yttrium chelates with serum has been studied by the method of pressure filtration for determining the relative binding strength of chelating agents in physiological media. The filterability of yttrium from serum and serum filtrate was found to be related to the stability constants of the yttrium chelates. The data obtained in pressure filtration studies are in agreement with those obtained in excretion studies carried out in experimental animals and man. Equilibrium dialysis studies of the binding of yttrium chelates to albumin and γ-globulin have shown that binding of albumin occurred with only two of the yttrium chelates, ethylenediamine di(O-hydroxy-phenylacetate), Y-EDDHA, and *N*, *N*-di(2-hydroxyethyl) glycine, Y-DHEG. The interaction of yttrium-EDDHA with albumin was further studied and this reaction was found to be reversible. An explanation for the protein binding of this yttrium chelate was proposed.

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EFFECT OF EXCESS CHELATING AGENTS ON RARE-EARTH DECONTAMINATION*

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The affinity of chelating agents for heavy metals and the rare earths has motivated their study in efforts at stable and radioactive metal detoxification. In general, it has been found that metabolically inert chelating agents having strong metal affinities are effective in enhancing excretion of the unwanted metals. That the actual mechanism of removal may be complex was suggested by the fact that in spite of the quantitative removal of C^{14}-labeled Ca-EDTA, only 10 per cent of La^{140} was excreted following its intravenous administration as the relatively stable metal chelate La-EDTA. In an effort to understand better the nature of the processes involved, it was decided to study the metabolism of lanthanum and yttrium chelates having a wide range of metal chelate stabilities in man and for which the 24-hour cumulative urinary excretion of the metal label following intravenous tracer administration ranged from 5 per cent to 100 per cent.

In attempting to construct a phenomenological description of the metabolism of small, highly diffusible molecules such as metal chelates, it is apparent that diffusion effects and intracompartment concentration gradients will play a prominent role.[1,2] That these concentration gradients may give rise to "spurious" exponential terms has been pointed out previously.[3] In spite of these difficulties, it is claimed by Bauer and Ray[4] that it is possible to derive uniquely an interpretation of strontium metabolism a priori from various strontium data while Lewallen *et al.*[5] somewhat more conservatively describe iodoalbumin metabolism in terms of a range of permissible models. Since the straightforward multicompartment approach was believed unsuited for our work because of the presence of diffusion effects, the lack of access to various compartments, the essential complexity of any remotely reasonable model, the inherent experimental errors and biological variability that limit the accuracy of the higher order determinants in multicompartment theory, it was decided to compensate for some of these difficulties by transforming the initial steady-state 4-compartment model with intracompartment diffusion gradients into a diffusion-compensated nonsteady-state 2-compartment system. This was done by combining the experimental results of several studies, each of which was designed to measure one, or at most two, of the unknown parameters as functions of time. Since the problems encountered and the methods employed in this series of studies might be pertinent in other areas of investigation, a rather detailed description of the approach will be given.

Since large amounts of chelating agent are employed in metal decontamination, it was also decided to study how the excretion of metal chelates depended

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upon the amount of excess chelating agent present. A theory involving coupled differential equations will be proposed to explain the observed results.

Materials and Methods

A series of yttrium chelates (Y-NTA, yttrium nitrilotriacetate, Y-HEEDTA, yttrium N (2-hydroxyethyl) etyhlenediaminetriacetate, Y-BAETA, yttrium bis (2-aminoethyl) ether tetraacetate, Y-EDTA, yttrium ethylenediaminetetraacetate, Y-CDTA yttrium cyclohexane *trans*-1,2-diaminetetraacetate, and Y-DTPA, yttrium diethylenetriaminepentaacetate) were injected intravenously at tracer levels to man and the subsequent urinary excretion of yttrium and yttrium plasma levels studied by means of a Y^{90} label at 1, 4, 8, and 24 hours following the injection of the tracer. For the tracer studies, the doses consisted of 1 mg. Y combined in a metal to chelate ratio of 1.0 to 1.2.

When studying the effect of excess chelating agent, an attempt was made to inject the tracer and carrier chelate in less than 10 min. The general procedures and methods followed throughout in this work parallel closely the techniques reported earlier.[6]

System I

A model for yttrium metabolism following intravenous administration as yttrium EDTA is shown in FIGURE 1. Compartment 1 represents yttrium as the metal chelate in the vascular compartment, Compartment 2 represents yttrium in nonchelated form in the vascular compartment, Compartment 3 represents diffusible extravascular yttrium in chelated form, while Compartment 4 represents extravascular yttrium in nonchelated form. The arrows, of course, represent transfers between the indicated compartments.

On examination, it is seen that ϵ, representing tissue deposition, can be neglected because the chelating agents are themselves metabolically inert and are quantitatively excreted; γ and γ' are also negligible at tracer levels both on experimental grounds and, theoretically, because chelate metal recombination is then a process of second order smallness. Furthermore, it is assumed that the rate of metal chelate dissociation β or β^1 is the same throughout the system.* The resulting model is shown in FIGURE 2.

It has been shown that the experimental accuracy required to analyze properly a system such as FIGURE 2 is often unattainable, and that diffusion effects may drastically interfere with correct interpretations.[3] Therefore in the next model we do not consider vascular-extravascular transfers but rather employ a simplified description (FIGURE 3). Here the complete system is represented by two chemical compartments and no information is directly sought about geographical location.

The system can be represented algebraically thus:

$$\frac{dy}{dt} = -\beta y - \alpha y =$$

rate of change of Y in Compartment 1. (1)

* That this is not always true is suggested by the local deposition of yttrium in the kidney cortex following administration of yttrium-HEIDA.

$$\frac{dY'}{dt} = -\alpha' Y' - \epsilon' Y' + \beta Y =$$

rate of change of Y′ in Compartment 2. It should be noted that in FIGURE 3 and EQUATION 1, α, α' and ϵ' are no longer constants since the rates of urinary

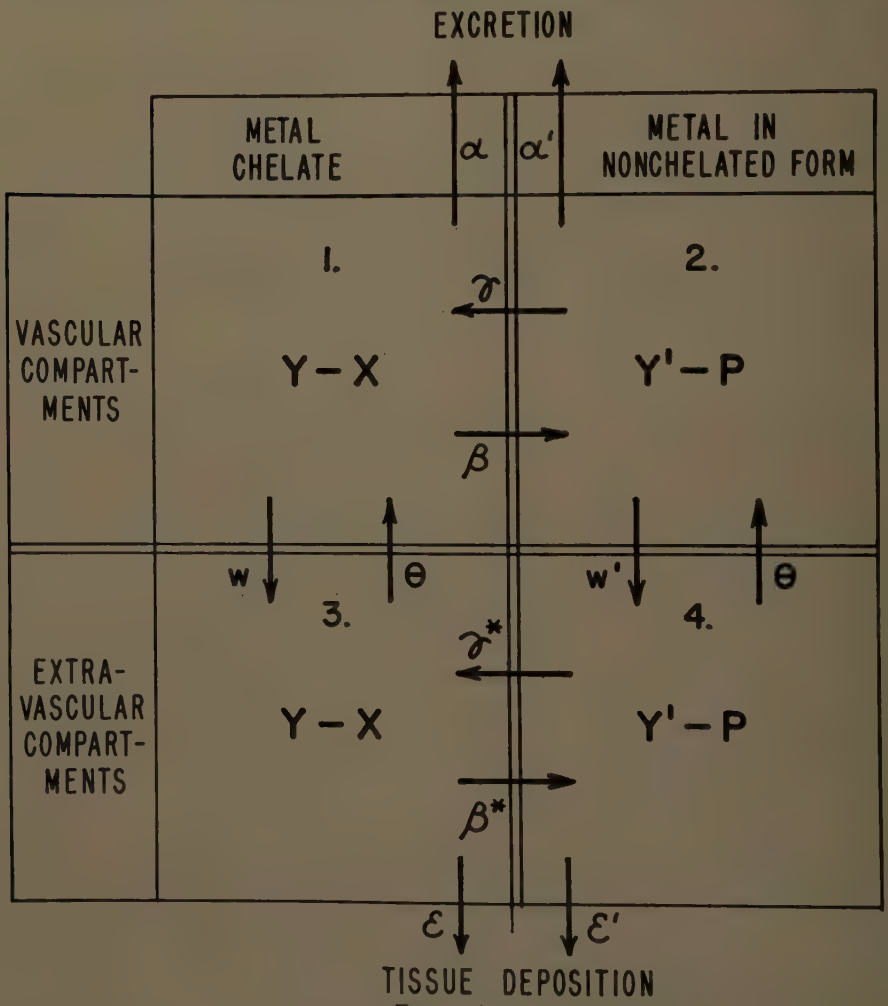

FIGURE 1.

excretion and tissue deposition must now necessarily depend indirectly upon the relative geographical distribution of the metal. That is, if most of the yttrium is in the vascular system then the value of α must be relatively high. As the geographical distribution of the yttrium changes, the values of α, α' and ϵ' change even though the physiological mechanisms remain constant and unaltered.

Since it is found experimentally that yttrium administered in weakly chelated form is excreted relatively poorly, it follows that $\alpha' \ll \alpha$. Since the period of very rapid geographical equilibration is ~10 min. and since, in this period, the

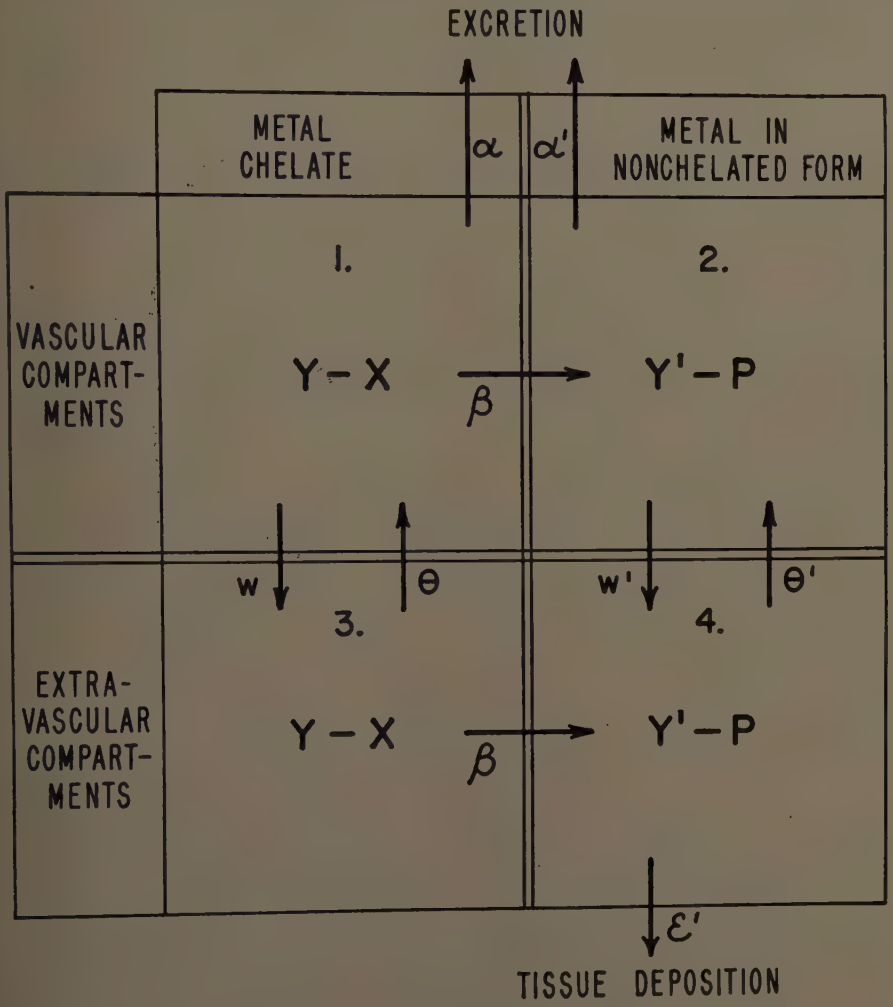

FIGURE 2.

yttrium is majorly chelated (for all but the weakest chelates) little error is introduced by considering α' and ϵ' constant.

Assuming that yttrium in Compartment 2 is truly dissociated and no longer reflects the particular form in which it was administered, α' and ϵ' should be the same for all studies. Furthermore, since the chelating agents are essentially small, inert molecules, it seems reasonable to assume α is approximately the same function of time for all chelates although preliminary studies with C^{14}-labeled chelates indicate that small differences may exist.

Instead of evaluating the three parameters β, α', and ϵ' and the function $\alpha(t)$, which in a single experiment would still give rise to formidable numerical difficulties, it proves easier to treat initially certain special cases. If $\alpha \ll \beta$ (that is, for weak yttrium chelates such as Y-NTA), chelated yttrium rapidly disappears and the model of FIGURE 3 reduces to that of FIGURE 4.

The 24-hour cumulative urinary excretion in per cent of dose is

$$U_{24} = \int \alpha' Y' \, dt \approx \frac{\alpha'}{\alpha' + \epsilon'} \times 100 \qquad (2)$$

From the slope of the plasma disappearance curve, which approximates $\alpha' + \epsilon'$ and the value of U_{24}, it follows that α' and ϵ' can be determined.

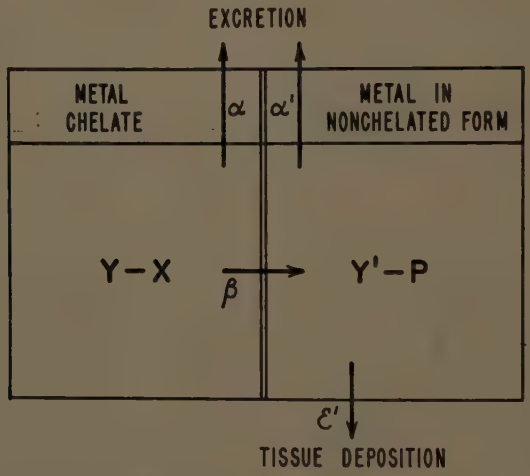

FIGURE 3.

To evaluate $\alpha(t)$ a very strong metal chelate is employed and, consequently, α', ϵ', and β can all be neglected. EQUATIONS 1 reduce to

$$\frac{dY}{dt} = -\alpha Y \qquad (3)$$

From EQUATION 3 and the experimental rate of yttrium urinary excretion, $\alpha(t)$ can be numerically approximated.

Having evaluated α', ϵ', and $\alpha(t)$, using very weak and very strong chelates, it is now possible to study intermediate strength metal chelates. The only unknown is then the single parameter, the dissociation rate α, which is chosen to satisfy best the 1-, 4-, 8-, and 24-hour cumulative urinary excretion values.

System II

The use of large molar excesses of chelating agents is customary in metal detoxification. In an effort to analyze the process mathematically, so as to be able to predict response, a series of studies were carried out in which 1-mg. quantities of yttrium labeled with Y^{90} and combined with ~1, 100, 500, 1500 mg. of excess chelating agent, usually as the calcium chelate, were successively injected intravenously to the same patient.

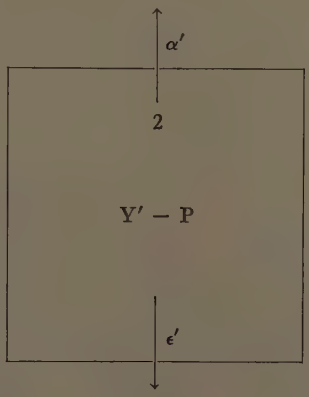

FIGURE 4.

If Y represents chelated yttrium; Y′, nonchelated yttrium; X, excess chelating agent; and γ, a constant representing recombination probability, then the differential equations describing the system are*

$$\frac{dY}{dt} = -\beta Y - \alpha Y + \gamma XY'$$
$$\frac{dY'}{dt} = -\alpha' Y' - \epsilon' Y^1 + \beta Y' - \gamma XY' \qquad (4)$$

Comparing EQUATIONS 1 and 4, the additional terms $\gamma XY'$ represent recombination of Y′ with X to form Y.

It can be shown that if X is proportional to $e^{-\alpha t}$ both Y and Y′ satisfy second order differential equations of the form

$$\frac{d^2Y}{dt^2} + (a + be^{-t})\frac{dY}{dt} + (c + de^{-t})Y = 0 \qquad (5)$$

The solutions to EQUATION 5 are not readily obtained by analytical methods,[3] although numerical integration procedures can be used if necessary. Nevertheless the general dependence of yttrium urinary excretion on the amount of excess chelating agent present in the dose can be demonstrated. To do this

* It is assumed throughout that the chelates are metabolically inert and do not effect any physiological parameters as such. A more general treatment of the "tracer analysis" of nontracer doses will appear elsewhere.[7]

we note that at any instant the amount of chelated yttrium being dissociated is βY. If it is assumed that the number of physiological binding (irreversible trapping) sites is always large for the amount of yttrium present and that whatever recombination is going to take place takes place quickly, then the chance of this dissociated yttrium being rechelated rather than irreversibly trapped is

$$\frac{\gamma e^{-\alpha t}}{K + \gamma e^{-\alpha t}} \tag{6}$$

where $\gamma e^{-\alpha t}$ is proportional to the amount of excess chelating agent remaining

$$\frac{u-u_0}{y_0} = \frac{(1+y)^m}{\gamma^m} \int_x^{m+1} (1-x/z)^m dx \quad ; \quad y = \gamma/x$$

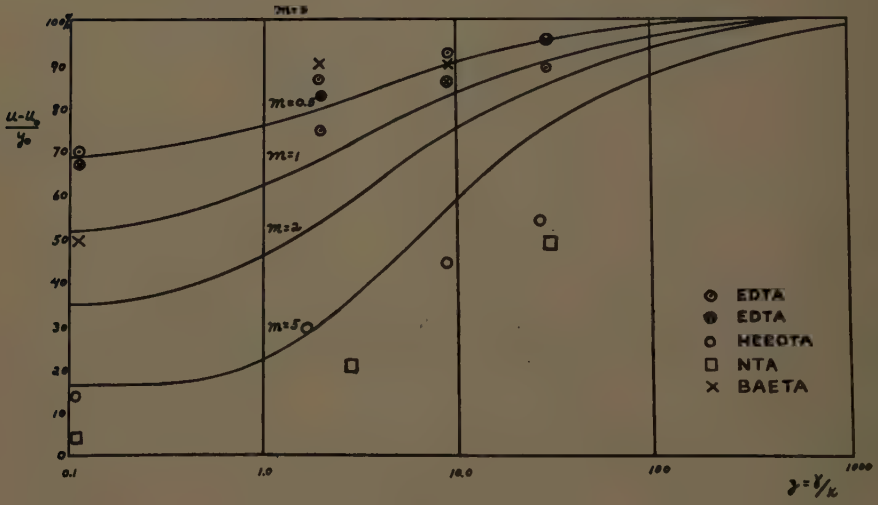

FIGURE 5.

at time t and the constant K is a measure of the physiological trapping power. On the basis of the above assumptions

$$\frac{dY}{dt} = -\beta Y - \alpha Y + \frac{\gamma e^{-\alpha t}}{K + \gamma e^{-\alpha t}} \beta Y \tag{7}$$

Using EQUATION 7, it is shown in APPENDIX II that the 24-hour cumulative urinary excretion of yttrium chelate can be approximated by:

$$\frac{U - U_0}{Y_0} = \frac{(1+z)^M}{\gamma_z{}^M} \int_K^{K+\gamma} \left(1 + \frac{K}{x}\right)^M dx; \qquad z = \gamma/K, M = \frac{\beta}{\alpha} \tag{8}$$

The definite integral can be readily evaluated for any integral value of M.

A series of studies have been carried out using EDTA, HEEDTA, NTA, BAETA, and DTPA. The theoretical curves and experimental points are

shown in FIGURE 5. It should be noted that in agreement with experiment, the theory predicts relatively little change in the cumulative urinary excretion so long as the chelating agent is present in less than 10-mg. quantities, although the metal-chelate molar ratios change by a factor of 10. There is no simple dependence of the urinary excretion upon the metal-chelate molar ratio such as might be expected by mass-action considerations during equilibrium conditions. It is believed that for high stability compounds ($\ln K \gtrsim 20$) at low concentrations ($\lesssim 10^{-6}\ M$) the considerable time required for chemical equilibrium to occur makes the use of differential equations preferable to simple mass action principles in physiological analysis whenever the physiological processes occur during times roughly comparable to or less than the time required for chemical equilibration.

APPENDIX I

Determination of $\alpha(t)$

To find $\alpha(t)$ we assume that it is sufficiently accurate to characterize average values for the intervals 0–1, 1–4, 4–8, and 8–24 hours. These average values α_{a-b} should be appropriate for all nonmetabolized, sufficiently stable metal chelates (such that $e^{-\beta t} \approx 1$ throughout the period of rapid diffusion $\sim$10 min.). A separate analysis is necessary, however, for moderately weak metal chelates such as Y-HEEDTA. Substituting the average experimental values of the urinary excretion following the administration of Y-EDTA with a large excess of Ca-EDTA in EQUATION 1A.

$$U_{a-b} = \int_a^b Y_{0a}\alpha_{a-b}e^{-\alpha_{a-b}t}\,dt \qquad (1A)$$

where U_{a-b} is urine collected between time a and time b, Y_0 is the injected dose, Y_{0a} is the amount remaining after time a, or $Y_{0a} = Y_0 - U_{0-a}$, and α_{a-b} is the average value of α in the time interval from a to b. It follows that:

TIME INTERVAL IN HOURS

	0–1	1–4	4–8	8–24
U_{a-b} =	35%	30%	15%	15%
α_{a-b} =	0.43	0.21	0.15*	0.09*

* These values assume that the yttrium remaining after 4 or 8 hours is still in diffusible form; the actual value of α for diffusible material in this patient is probably higher $\sim$0.20, since the 24-hour cumulative urinary excretion is only $\sim$90–95%.

Determination of Trapping and Excretion "Constants" for Nonchelated Yttrium

To find the rates of trapping and excretion of nonchelated yttrium, the experimental values of the urinary excretion of yttrium following the administration of the very weak metal chelate Y-NTA are substituted in EQUATION 2A below, and the values of α' and ϵ' determined.

$$U'_{0-b} = \int_0^{t_b} Y_0 \alpha'_{0-t_b} e^{-(\alpha'_{0-t_b}+\epsilon'_{0-t_b})t}$$

$$= \frac{Y_0}{1 + \frac{\epsilon'_{0-t_b}}{\alpha'_{o-t_b}}} [1 - e^{-(\alpha'_{0-t_b}+\epsilon'_{0-t_b})t_b}] \qquad (2A)$$

In solving EQUATION 2A for α' and ϵ' it is convenient to assume initially that they are constants. Letting t_b equal 24 hours, the exponential term in 2A becomes insignificant, and it follows that $1/[1 + (\epsilon'/\alpha')] \approx 0.07$. TABLE 2A below shows the calculated value of the sum of α' and ϵ' following the substitution of the indicated experimental values of the urinary excretion in EQUATION 2A.

	0–1	0–2	0–3	0–4	0–8
U_{0-b}	1.60%	3.38%	4.08%	4.62%	5.19%
$(\alpha' + \epsilon')_{0-t_b}$	0.26	0.33	0.30	0.27	0.18

It is apparent that α' and ϵ' are actually variables but since, as the table shows, relatively little variation occurs in the first 4 hours and since, for the strong chelates at least, the excretion of nonchelated yttrium is small, little error is introduced in assuming constancy. Therefore, solving EQUATION 3A below,

$$\frac{1}{1 + \frac{\epsilon'}{\alpha'}} \approx 0.07; \qquad \alpha' + \epsilon' = 0.27 \text{ (4-hour value)} \qquad (3A)$$

it follows that:

$$\alpha' \approx 0.019; \qquad \epsilon' \approx 0.25$$

Determination of the Urinary Excretion of Chelated Yttrium

Since α', ϵ', and $\alpha(t)$ have all been approximated, the only remaining parameter is β. The choice of β is arbitrary, but if the previous assumptions are correct, then a single constant value of β should permit prediction of the complete pattern of urinary excretion following the injection of an yttrium chelate. Assuming that the dissociation constant for Y-EDTA is $\beta \approx 0.20$, and substituting the appropriate value of α_{a-b} in EQUATION 4A below;

$$U_{a-b} = \int_0^{|b-a|} \alpha_{a-b} Y_{0a} e^{-(\alpha_{a-b}+\beta)t}\, dt \qquad (4A)$$

a predicted rate of urinary excretion is obtained as tabulated below.

	0–1	1–4	4–8	8–24
U_{a-b}	32%	19%	6%	2%

Determination of the Amount of Nonchelated Yttrium Present as a Function of Time

The differential equation describing the amount of nonchelated yttrium in diffusible form is

$$\frac{dY'_{a-b}}{dt} = -\alpha' Y'_{a-b} - \epsilon' Y'_{a-b} + \beta Y_{0a} e^{-(\alpha_{a-b}+\beta)t} \tag{5A}$$

having the solution

$$Y'_{a-b} = \left[Y'_{0a} - \frac{\beta Y_{0a}}{(\alpha' + \epsilon') - (\alpha_{a-b} + \beta)} e^{-(\alpha'+\epsilon')t} + \frac{\beta Y_{0a} e^{-(\alpha_{a-b}+\beta)t}}{(\alpha' + \epsilon') - (\alpha_{a-b} + \beta)} \right] \tag{6A}$$

where Y_{0a} and Y'_{0a} are calculated from the previous interval using each time the appropriate value of α_{a-b}.

The table below indicates the calculated amount of yttrium present in chelated and nonchelated form at the beginning of each time interval.

	0–1	1–4	4–8	8–24
Y_{0a}	Y_0	$0.53Y_0$	$0.19Y_0$	$0.05Y_0$
Y'_{0a}	0	$0.13Y_0$	$0.17Y_0$	$0.10Y_0$

Determination of the Urinary Excretion of Nonchelated Form

The urinary excretion of nonchelated yttrium is given by:

$$U'_{a-b} = \int_{t=a}^{t=b} \alpha'_{a-b} Y'_{a-b}\, dt \tag{7A}$$

Substituting EQUATION 6A in EQUATION 7A, it follows that:

$$U'_{a-b} = \frac{\alpha'}{\beta' + \epsilon'} \left[Y'_{0a} - \frac{\beta Y_{0a}}{(\alpha' + \epsilon') - (\alpha_{a-b} + \beta)} \right] (1 - e^{-(\alpha'+\epsilon')t}) + \frac{\alpha'}{\alpha_{a-b} + \beta} \left[\frac{\beta Y'_{0a}}{(\alpha' + \epsilon') - (\alpha_{a-b} + \beta)} \right] (1 - e^{-(\alpha_{a-b}+\beta)t}) \tag{8A}$$

Substituting the appropriate values for each time interval in EQUATION 8A, the tabulated values are obtained

	0–1	1–4	4–8	8–24
U^1_{a-b}	0.1%	2.9%	1.1%	~0.9%*

* Assuming $\alpha_{8-24} \approx 0.20$.

Finally, the total theoretical yttrium excretion is obtained by adding the two

separate contributions, namely U_{a-b} and U^1_{a-b}, and the results compared with experiment below.

Time interval	U_{a-b}	U^1_{a-b}	Total theor. cum. ur. excret.	Exp. value
0–1	32%	1.0%	33.0	33.2
1–4	19	2.9	54.9	57.7
4–8	6	1.1	62.0	62.3
8–24	2	0.9	64.7	64.5

Appendix II

As indicated in the text,

$$\frac{dY}{dt} = -\alpha Y - \beta Y + \frac{\gamma e^{-\alpha t}}{K + \gamma e^{-\alpha t}} \beta Y. \tag{1B}$$

Integrating, assuming the constancy of α*, and letting $Y = Y_0$, $U = U_0$ at the time $t = 0$, it follows that:

$$Y = \frac{Y_0(\gamma + K)^{\beta/\alpha} e^{-\alpha t}}{(\gamma + Ke^{\alpha t})^{\beta/\alpha}} \tag{2B}$$

Now

$$U = \int_{t=0}^{t} \alpha Y \, dt = Y_0(\gamma + K)^{\beta/\alpha} \int_0^{24\text{hrs}\sim\alpha} \frac{\alpha e^{-\alpha t} \, dt}{(\gamma + Ke^{\alpha t})^{\beta/\alpha}} \tag{3B}$$

let

$$X = \gamma e^{-\alpha t} + K \tag{4B}$$

Substituting in EQUATION 3B and simplifying

$$\frac{U - U_0}{Y_0} = \frac{(1 + z)^m}{\gamma z^m} \int_K^{K+\gamma} \left(1 - \frac{K}{X}\right)^m dx; \qquad z = \frac{\gamma}{K}, \qquad m = \frac{\beta}{\alpha} \tag{5B}$$

The integral in EQUATION 5B can be expressed as analytic functions of z for integral values of m. This dependence is illustrated in FIGURE 5 of the text for $m = 0$, 0.5, 1, 2, and 5.

In comparing the predicted values of the 24-hour cumulative urinary excretion with experimental results, a correction must be made for the small amount of nonchelated yttrium excreted. Since yttrium administered as a Y-NTA tracer is excreted ~5 to 8 per cent, it is assumed that

$$U_{\text{total}} = U_{\text{chelated yttrium}} + \underbrace{0.06(100 - U_{\text{chelated yttrium}})}_{\substack{\text{excreted as nonchel.} \\ \text{yttrium}}}$$

* Although $\alpha(t)$ is variable, the error introduced in integrating EQUATION 7B should not be an important factor in investigating the dependence of U upon the amount of excess chelate present (that is, $U = U(z)_0$) α can be considered to be an average value for a given patient and a given chelating agent.

or that

$$U_{\text{chelated yttrium}} = \frac{U_{\text{total}} - 6}{0.94} \qquad (6B)$$

The approximate correspondence between the values of β and γ derived in the analysis of APPENDIX I and the values of the ratio $m = \beta/\alpha$ for the various chelating agents is encouraging. Exact correspondence should not, of course, be expected since, although the studies analyzed in APPENDIX I were all done on the same patient, the excess chelate experiments employed a different patient for each chelate studied, with the kidney clearance parameter α necessarily varying from patient to patient.

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THE MOBILIZATION AND EXCRETION OF LEAD IN CATTLE: A COMPARATIVE STUDY OF VARIOUS CHELATING AGENTS*

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The principle of chelation has found application in the treatment of poisoning by heavy metals. The role of chelating agents in this regard is to form with the metal a diffusible complex that is readily excreted from the body. Numerous factors determine how readily this end is attained. Some involve the interaction between the metallic ion and the ligand. Others involve interactions between the animal and the ligand. A given chelating agent may have a strong affinity for a metal but be useless as a detoxifying agent for reasons of rapid metabolism *in vivo* or toxicity to the animal related or unrelated to chelation phenomena. It is therefore necessary to approximate the conditions of intended use in order to evaluate chelating agents as detoxifying drugs.

Our interest in chelation phenomena concerns the treatment of lead poisoning. A number of chelating agents have been used to accelerate the excretion of lead from the body. The calcium complex of disodium ethylenediaminetetraacetate (Ca EDTA) is generally considered to be the chelating agent of choice for the treatment of lead poisoning. Our experiences with the use of this compound in the treatment of lead poisoning in cattle have been disappointing.[1] The slow and incomplete removal of lead from the blood during and following intensive therapy suggested that the process of detoxification should be studied.

Methods

Female Holstein-Friesian calves approximately 2 to 4 months of age were used in these studies. Lead was administered orally in individual doses of 0.25–1.0 gm. as the nitrate at 1- to 3-day intervals until a concentration of 0.3–1.0 ppm lead in the blood was attained. Experimental studies were always begun 3 to 5 days following the last dose of lead. In only one case did clinical signs of lead poisoning result. All other animals remained in apparent good health during and following all experimental trials.

During the twenty-four hour period preceding the administration of chelating agents the base line rate of lead excretion in urine and feces and the rate of decrease of lead in the blood were established. Chelating agents were administered by intravenous infusion over a period of ten minutes.

The analysis of samples for lead was made with acid oxidizing agents for the digestion of biomaterials and a standard dithizone spectrophotometric procedure.[2]

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The Effect of Ca EDTA on the Mobilization and Excretion of Lead

Eight separate experiments were conducted on 8 calves using a dose of 110 mg./kg. Ca EDTA in each case. The excretion of lead in the urine was greatly accelerated in every case. This effect persisted for at least 48 hours following the administration of Ca EDTA (FIGURE 1). A pronounced, rapid

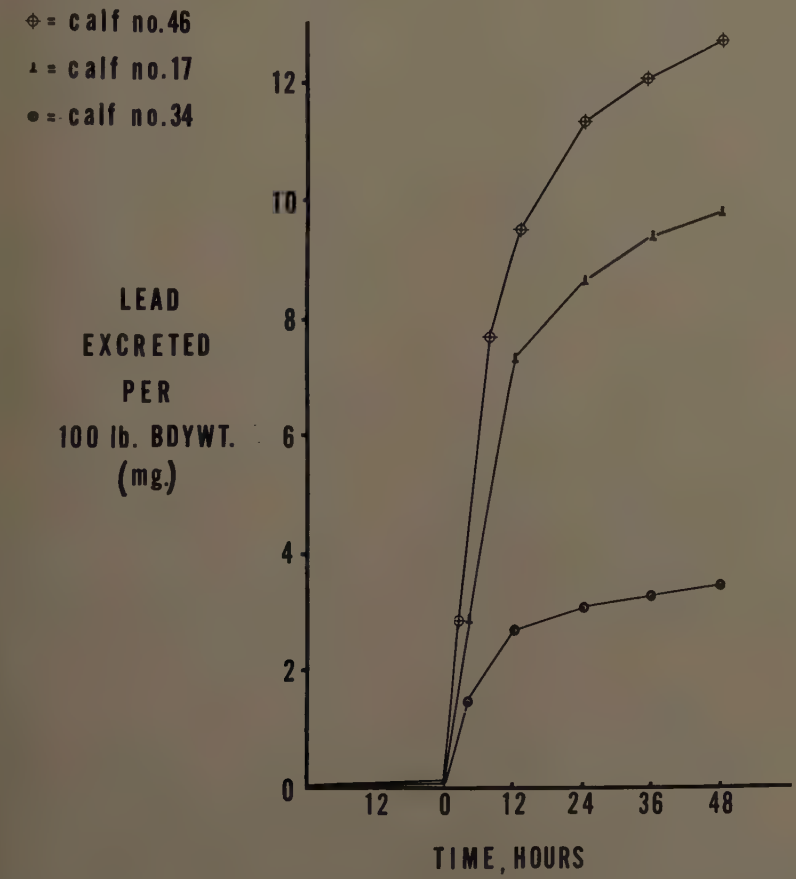

FIGURE 1. The effect of 110 mg./kg. Ca EDTA on the cumulative excretion of lead in the urine. Ca EDTA was injected at zero hour.

rise in the concentration of lead in the blood plasma always occurred. This was accompanied by a moderate, progressive decrease in the concentration of lead in erythrocytes (FIGURE 2). There appeared to be a positive correlation between the concentration of lead in erythrocytes at the initiation of an experiment and the amount of lead excreted in the urine during the 48-hour period following the administration of 110 mg./kg. Ca EDTA. This relationship is readily apparent in the case of the 3 animals selected for illustrative purposes (FIGURES 1 and 2). A regression line was fitted using the data obtained in all 8 calves by the method of least squares and a 95 per

cent confidence band for any new observation was obtained[3] (FIGURE 3). In this graphic representation, the amount of lead excreted is per 100 lb. body weight. The inference drawn was that there exists a steady state relationship between the concentration of lead in erythrocytes and the amount which can be mobilized and excreted in the urine by Ca EDTA. Only approximately 10 per cent of the lead excreted in the urine could be accounted

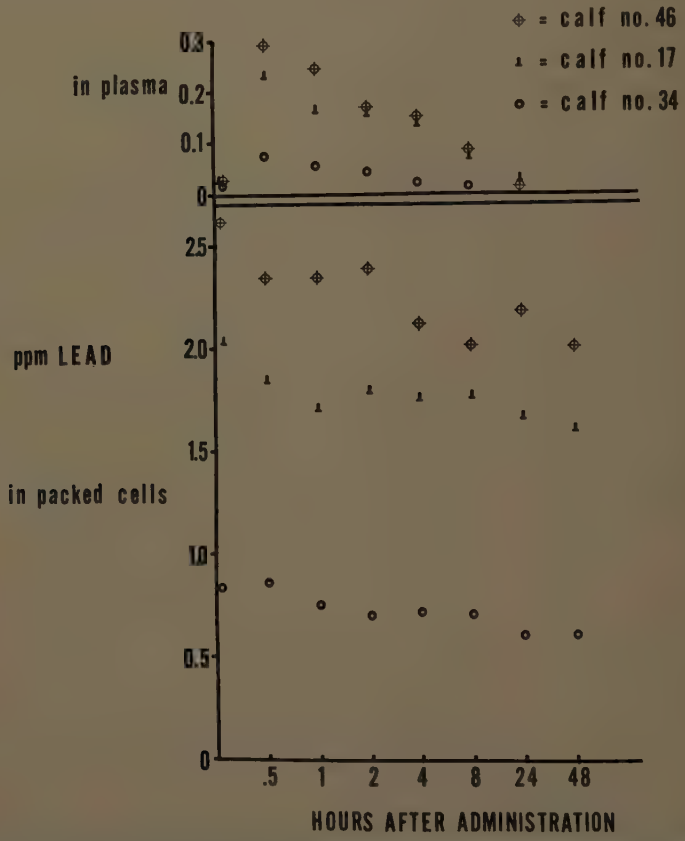

FIGURE 2. The effect of 110 mg./kg. Ca EDTA on the concentration of lead in erythrocytes and in plasma. Ca EDTA was injected at zero hour.

for by the decrease in lead associated with the erythrocytes. The remaining 90 per cent presumably originated from tissues other than the blood. Since the toxic effects of lead are presumed to result from its presence at critical points in tissues other than erythrocytes or bone, it was desirable to gain some indication that changes in the concentration of lead in blood reflected similar changes in other tissues. The simultaneous determination of lead in the blood and in the liver of 1 calf on several occasions during intermittent treatment with multiple doses of Ca EDTA (110 mg./kg./dose) suggested that this is true (FIGURE 4).

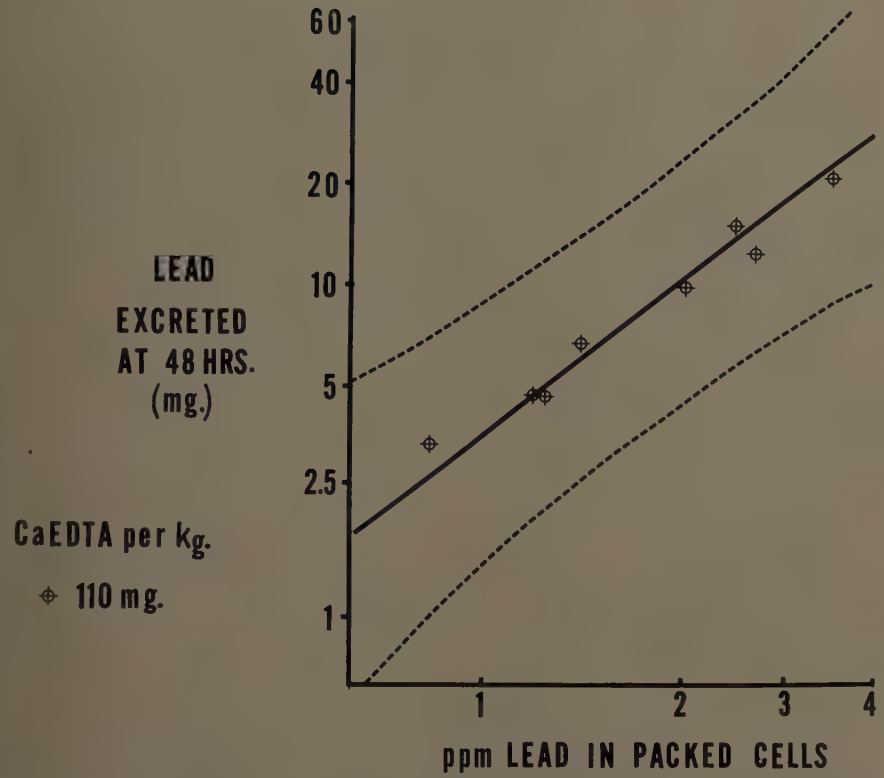

FIGURE 3. The relationship between the concentration of lead in erythrocytes prior to Ca EDTA administration and the amount of lead excreted in the urine during the succeeding 48 hours. Lead excretion is reported on the basis of mg./100 lb. body weight. The solid line is the regression line for the data. The hatched lines represent the 95 per cent confidence interval for any new observation.

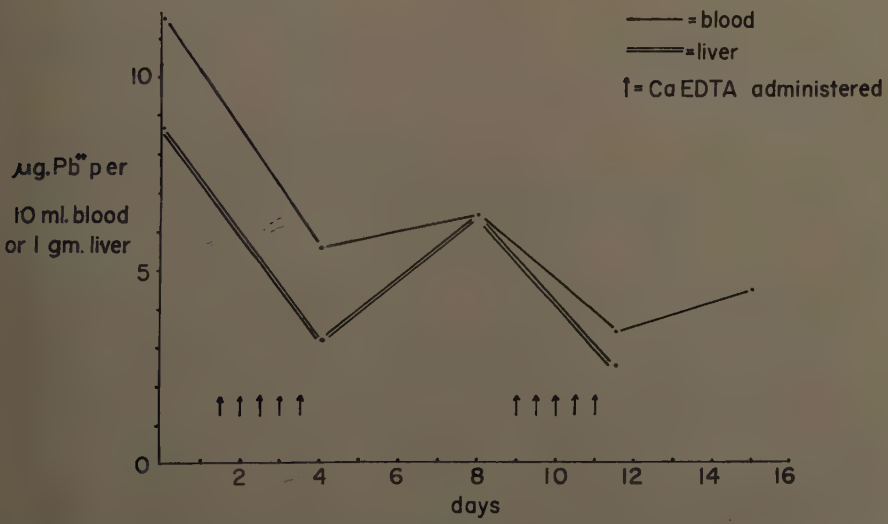

FIGURE 4. The effect of Ca EDTA on the concentration of lead in the blood and liver of a calf. Each dose of Ca EDTA was 110 mg./kg.

The apparent inability of Ca EDTA to effect more than a partial removal of lead from soft tissue following the administration of multiple doses suggested that a maximal response was being obtained with the 110 mg./kg. dose. In a series of individual experiments performed as in the 110 mg./kg. series, the dose of Ca EDTA was varied. The results vis à vis urinary excretion of lead suggest that 110 mg./kg. did indeed approximate maximal response at least for our experimental conditions (FIGURE 5). The incomplete

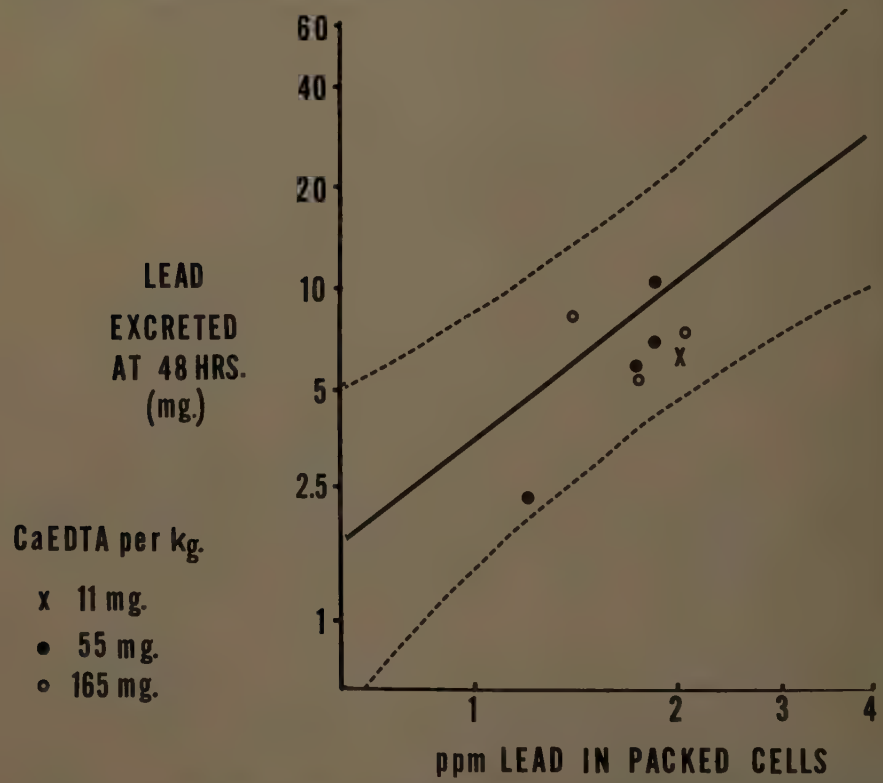

FIGURE 5. The effect of a range of Ca EDTA doses on the excretion of lead in the urine. Each point plotted depicts the cumulative excretion of lead in the urine for one calf. The regression line and confidence interval are those for 110 mg./kg. Ca EDTA. Lead excretion is reported on the basis of mg./100 lb. body weight.

removal of lead from the soft tissues was evident from data obtained concerning the amount of lead remaining in the body at the conclusion of one excretion trial (TABLE 1). It was difficult to assess the importance of the gastrointestinal tract as a route for the elimination of the Pb EDTA complex. Appreciable and variable quantities of lead appeared in the feces even in the case of control calves to which no chelating agent was administered. The problem of distinguishing between lead being excreted into the gastrointestinal tract and lead merely passing through without being absorbed could have been circumvented by administering the lead intravenously. We preferred to avoid this since it has been shown that the distribution of lead in

the body following intravenous administration is quite different from that obtained following oral administration.[5]

It was possible to establish that the kidney does play a major role in the clearance of the Pb EDTA complex from the body. This was done by comparing the rate of Pb EDTA excretion in urine to the rate of inflow by the intravenous route. A normal calf not previously dosed with lead was used for this purpose. A loading dose of 7.5 mg. lead covered by 60 times its molar equivalent of Ca EDTA was administered rapidly by vein. Constant infusion of this preparation was instituted immediately afterward and continued at a rate of 116 μg. Pb/min. for the succeeding 2½ hours. Bladder urine was collected at 20-minute intervals. Endogenous creatinine and Pb EDTA plasma clearance values were calculated. The rate of Pb EDTA output in urine was approximately 65 per cent the rate of input. These results suggest that the kidney is a major route of Pb EDTA excretion. The lead unaccounted for may have been excreted into the gastrointestinal tract or it may have simply represented diffusion from plasma into other tissues. The similarity between creatinine clearance values and Pb EDTA clearance values suggests that Pb EDTA clearance is by glomerular filtration (TABLE 2).

TABLE 1

THE DISTRIBUTION OF LEAD FOLLOWING CA EDTA (110 mg./kg.*)

Organ	Weight, kg.	Per cent bodywt.	Lead (ppm)	Total mg. lead
Muscle	30.6†	40.3†	0.06	1.84
Skeleton	13.4†	17.7†	3.66	49.04
Skin	6.50	8.6	0.52	3.38
Stomach and forestomachs	2.50	3.3	0.10	0.25
Intestines	3.77	5.0	0.38	1.43
Heart	0.50	0.7	0.06	0.03
Lungs	0.96	1.3	0.20	0.19
Liver	1.32	1.7	2.96	3.90
Kidneys	0.45	0.6	2.44 cortex 0.38 medulla	0.91‡
Spleen	0.18	0.2	0.40	0.07
Brain	0.26	0.3	0.14	0.04
Blood	5.30	7.0†	0.26	1.38
Intestinal content	1.91	2.5	1.28	2.44
Stomach content	8.90	11.7	0.84	7.48
Bone				49.04 mg.
Soft tissue				13.42 mg.
Gastrointestinal tract				9.92 mg.
Amount of lead excreted in urine during 48 hr. period following 110 mg./kg. Ca EDTA				9.53 mg.

* Calf sacrificed 48 hours following Ca EDTA.
† Estimated from human data.[4]
‡ Assume 80 per cent cortex, 20 per cent medulla.

The Effect of Other Polyaminoacetic Acids on the Mobilization and Excretion of Lead*

Ionic charge or lack of it has an effect on the pharmacological behavior of certain classes of drugs such as the barbiturates, presumably by affecting

* We are indebted to Murray Weiner and Martin Dexter, Geigy Chemical Corporation, Ardsley, N. Y., for providing these compounds.

the membrane-penetrance characteristics of the compounds. Two polyaminoacetic acids were selected which should combine with divalent cations to confer on the complexes a net charge of zero. These were ethylenediaminediacetic acid (EDDA) and *N*,*N*′-dihydroxyethylenediaminediacetic acid (HEDDA). Two others were selected for their high affinities for heavy metals. These were diethylenetriaminepentaacetic acid (DTPA) and cyclohexanediaminetetraacetic acid (CDTA). Both of these have higher stability constants for heavy metals than has EDTA. All 4 compounds were studied in single trials in the same manner as was Ca EDTA. The calcium complex was used in all cases in doses equivalent to 110 mg./kg. Ca EDTA.

The effect of these four chelating agents on the excretion of lead in the urine is indicated graphically (FIGURE 6). Ca EDDA appeared to be clearly inferior to Ca EDTA in this regard. The activities of Ca HEDDA, Ca

TABLE 2
THE RECOVERY OF LEAD IN URINE DURING CONSTANT INFUSION OF Pb EDTA*

Urine collection period (20–21 min. each)	Blood plasma concentration (interpolated)		Plasma clearance, ml./min./M²		Lead excreted/min. (μg.)
	Creatinine	Lead	Creatinine	Lead	
1	14.6	0.86	77	18	25
2	14.8	0.56	92	83	74
3	14.8	0.48	72	81	63
4	13.0	0.49	93	97	76
5	12.3	0.46	103	101	80
6	9.7	0.41	111	116	77
7	9.7	0.38	121	136	83
Average of last six collection periods					75.5

* Rate of lead infusion following loading dose = 116 μg./min.

CDTA, and Ca DTPA with respect to the urinary excretion of lead did not differ greatly from that generally obtained using Ca EDTA. The very respectable showing of Ca HEDDA was somewhat surprising in view of the inferior activity of Ca EDDA, the other diacetic acid screened. We have not found in the literature any stability constant data for this compound. The effect of Ca CDTA on the excretion of lead in urine suggests that further trials would be extremely unlikely to yield an average excretion of lead in urine superior to that obtained using Ca EDTA. By contrast, further investigation of Ca DTPA would have reasonable prospects in this regard. The distribution of lead in the blood was altered by these compounds in a manner very similar to that noted following the administration of Ca EDTA.

There is a tendency to anticipate and explain differences among related chelating agents, with respect to the removal of heavy metals from the body, on the basis of stability constants. If stability constants are the major factor involved, Ca DTPA would be expected to exhibit some degree of superiority over Ca EDTA, since its known stability constants are greater than are those of Ca EDTA for the same metals. The selectivity of a chelating agent prob-

ably is important where the object is to chelate one specific cation species among many present. Calcium and magnesium ions, which are present in high concentrations in the body, might effectively cancel the greater affinity of one agent over another for a heavy metal. Thus, although Ca CDTA

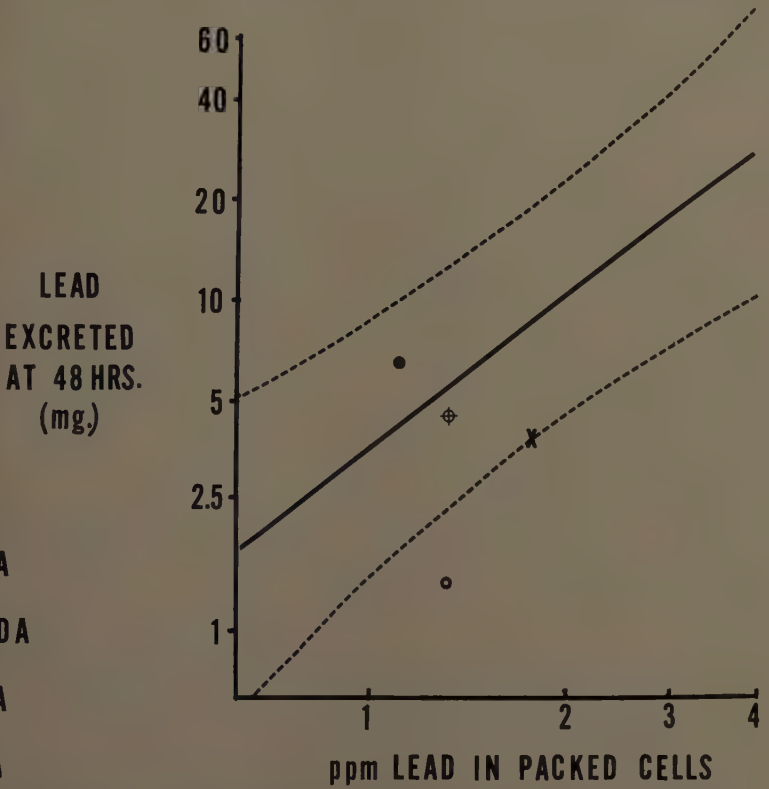

FIGURE 6. The effect of four polyaminoacetic acids on the excretion of lead in the urine. Each point plotted depicts the cumulative excretion of lead in the urine for one calf. All compounds were administered as the calcium chelates in doses equivalent to 110 mg./kg. Ca EDTA. The regression line and confidence interval are those for 110 mg./kg. Ca EDTA. Lead excretion is reported on the basis of mg./100 lb. body weight.

TABLE 3

STABILITY CONSTANTS* OF EDTA, CDTA, AND DTPA FOR Zn^{+2}, Pb^{+2}, Ca^{+2}, Mg^{+2} (Log K_{MKe})

	Zn^{+2}	Pb^{+2}	Ca^{+2}	Mg^{+2}	Log K_{PbKe} − Log K_{CaKe}	Log K_{PbKe} − Log K_{MgKe}
EDTA	16.1	18.2	10.6	8.7	7.6	9.5
CDTA	18.7	19.7	12.1	10.3	7.6	9.4
DTPA	18.2	18.9	10.6	9.0	8.3	9.9

* These figures were provided by Martin Dexter and Murray Weiner, Geigy Chemical Corp., Ardsley, N. Y.

has a greater affinity for lead than has Ca EDTA, it also has a greater affinity for both calcium and magnesium (TABLE 3). These relationships might account for the apparent failure of Ca CDTA to perform more effectively than Ca EDTA with regard to the excretion of lead in urine. The interpretation of stability constants may be further complicated by the fact that the acid dissociation constants of chelating agents differ and modify, at least to some degree, the relationship between K_{MKe} and $[M^{+n}/MK_e]$ when comparisons are made at a relatively fixed *p*H value such as that of body fluids.

The Effect of Dithiols on the Mobilization and Excretion of Lead

It has been variously reported that 2,3-dimercaptopropanol (BAL) is beneficial and harmful in the treatment of lead poisoning. It is known to enhance the excretion of lead and other heavy metals. Its effects on 4 calves were studied, using the same experimental procedure as had been used in the study of the polyaminoacetic acids. The dose used was the molar equivalent of 55 mg./kg. Ca EDTA rather than of 110 mg./kg. Ca EDTA. A study of the literature concerning the toxicity of BAL suggested that this reduction in dosage would be necessary. Even the dose used has pronounced but apparently transient toxic effects. Shortly after intravenous administration the animals became highly excited. Lacrimation, salivation, and rhinorrhea also were prominent. All signs of toxicity lasted approximately 30 min. and were quite similar in all cases.

A pronounced rise in the urinary excretion of lead resulted from the administration of BAL. The rate of excretion tapered off rapidly 12 hours after the administration of BAL. Nevertheless, it was still somewhat greater than base line even 48 hours following BAL in all cases. The cumulative excretion of lead in the urine following the administration of BAL was generally less than that noted when using Ca EDTA in an equivalent dose (FIGURES 5 and 7). The effect of BAL on the distribution of lead in the blood was strikingly different from that of Ca EDTA. A precipitous fall in the concentration of lead in erythrocytes followed by a partial rebound was noted in every case. This is represented graphically for one case (FIGURE 8). In this respect the action of BAL in calves resembles that reported in man.[6]

The ease with which BAL removed lead from erythrocytes suggested that it might differ from EDTA as to distribution characteristics and perhaps might exert a synergistic effect when combined with Ca EDTA. Three separate experiments were conducted in which 110 mg./kg. Ca EDTA was administered followed one-half hour latter by BAL. The resultant excretion of lead in urine was not significantly different from that obtained using Ca EDTA alone (FIGURE 7). The contour and magnitude of the plasma lead curve were similar to those obtained using Ca EDTA alone whereas the changes in the concentration of lead in erythrocytes were indistinguishable from those noted when using BAL alone.

Certain other dithiols were screened in single experiments using the molar equivalent of 55 mg./kg. Ca EDTA. These compounds were 2,3-dimercaptopropionic acid (BAL acid), ethane-1,2-dithiol,* propane-1,3-dithiol, and 2,5-

dithiahexane.* Of these, 2,5-dithiahexane (the methyl sulfide analogue of ethane-1,2-dithiol) and propane-1,3-dithiol had no detectable effect on either the concentration and distribution of lead in the blood or on the excretion of lead in the urine. Ethane-1,2-dithiol was unique in its action. Its effect on the concentration of lead in erythrocytes was very similar to that

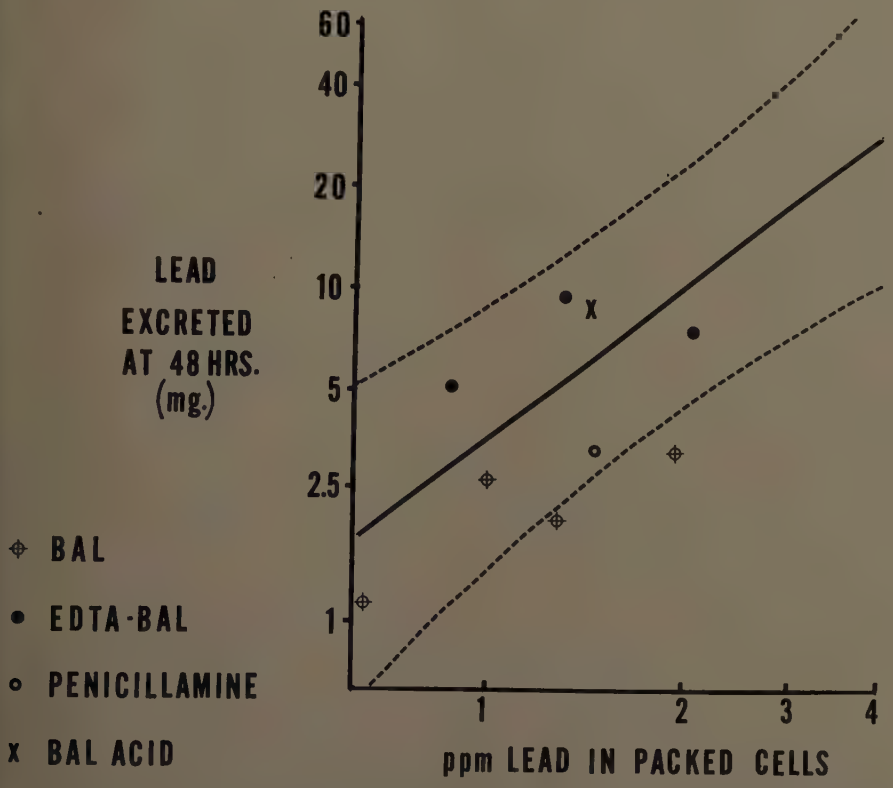

FIGURE 7. The effect of thiol-containing chelating agents on the excretion of lead in the urine. Each point plotted depicts the cumulative excretion of lead in the urine for one calf. All compounds were administered in doses equivalent to 110 mg./kg. Ca EDTA. The regression line and confidence interval are those for 100 mg./kg. Ca EDTA. Lead excretion is reported on the basis of mg./100 lb. body weight.

of BAL (FIGURE 8). The rebound effect was complete rather than partial as was the case with BAL. This rebound and the very small rise in the concentration of lead in plasma are perhaps consistent with the failure to enhance the excretion of lead in urine.

BAL acid appeared to be at least as active as Ca EDTA from the point of view of excretion of lead in the urine (FIGURE 7). In fact, at 48 hours postadministration the rate of lead excretion was greater than that generally

* We are indebted to W. W. Wannamaker, Wateree Chemical Co., Lugoff, S. C., for contributing ethane-1,2-dithiol and 2,5-dithiahexane.

observed using Ca EDTA. Its effects on the concentration and distribution of lead in blood were quite similar to those of Ca EDTA. It has previously been demonstrated that BAL acid is more toxic than BAL.[7] The effect of this compound was therefore observed in 2 rabbits prior to use in a calf. A dose equivalent to 110 mg./kg. Ca EDTA was administered to one rabbit

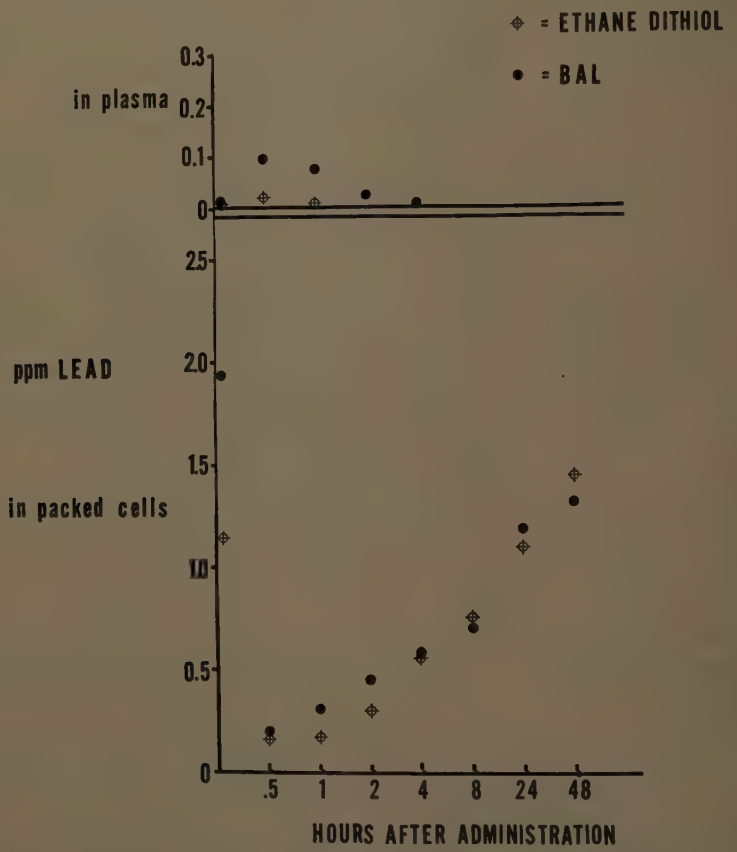

FIGURE 8. The effect of ethane-1,2-dithiol and of BAL on the concentration of lead in erythrocytes and in plasma of two calves. Compounds were administered in doses equivalent to 55 mg./kg. Ca EDTA at zero hour.

intravenously. No signs of toxicity developed during the thirty minutes following administration. At this time the animal became progressively more depressed, lapsed into a coma, and died. Toxic signs lasted approximately 30 min. The procedure was repeated using a second rabbit. In this instance calcium chloride was injected in a dose equivalent to that of BAL acid. This was done immediately following the BAL acid injection, using the intravenous route. No signs of toxicity developed. This suggested that the lethality of BAL acid was probably the result of calcium chelation. As

a result of this experience, the BAL acid to be administered to the calf was mixed with calcium chloride beforehand. No signs of toxicity developed during the first thirty minutes following the administration of BAL acid to the calf. At this time the familiar sequence of events previously seen using BAL was noted. Actually, the severity of excitement was greater than that associated with the use of BAL. A sedating dose of chloral hydrate was administered when matters appeared to be getting out of hand.

The most interesting observation in the dithiol series was the altered pattern of chelating activity resulting from the substitution of an acidic group for an alcoholic group in the BAL structure. This substitution appeared to enhance the overall chelating activity but to reduce cellular penetrance, at least insofar as erythrocytes were concerned. The superior de-leading activity of BAL acid as compared to BAL has previously been reported in rabbits.[7] The delayed nature of BAL acid toxicity could perhaps also be due to altered membrane penetrance characteristics resulting from the introduction of an acidic group.

The Effects of Aminothiol Compounds on the Distribution and Excretion of Lead

Interest in this group of compounds as therapeutic chelators has recently received considerable impetus with the discovery that 3,3-dimethylcysteine (penicillamine) enhances the excretion of copper, protects against experimental mercury poisoning, and enhances the excretion of lead.[8-10] Penicillamine was therefore screened for its activity as compared to that of Ca EDTA.* The racemic mixture of DL-penicillamine was used in a single experiment in a dose equivalent to 110 mg./kg. Ca EDTA. The effect of this compound on the cumulative excretion of lead in the urine was of the same order of magnitude as was that of Ca EDTA (FIGURE 7). Forty-eight hours following administration, the rate of excretion of lead in the urine was still 3 times base line. The effect of penicillamine on the distribution of lead in the blood was quite similar to that of Ca EDTA. One other compound having the general structural feature $R \cdot CH(SH) \cdot CH(NH_2) \cdot R$ was screened. This compound, 2-aminoethanethiol, has not, to our knowledge, been characterized as to its ability to alter the rate of excretion of heavy metals. It was administered at a dose equivalent to 110 mg./kg. Ca EDTA. It had no noticeable effect on either the concentration and distribution of lead in the blood, or on the excretion of lead in the urine. This total lack of activity was somewhat surprising in view of the relatively high stability constants that have been reported for the cobalt, zinc, and nickel chelates.[11] No toxic effects were noted following the administration of either of these two compounds.

The Effects of Sodium Citrate and of Catechol-1,3-Disulfonate on the Mobilization and Excretion of Lead

Sodium citrate was screened because it has been reported to accelerate the excretion of lead in the urine.[12] The dose used was equivalent to 110

* We are indebted to L. Michaud of Merck Sharp and Dohme Research Laboratories, Rahway, N. J., for providing the DL-penicillamine used in these studies.

mg./kg. Ca EDTA. It was mixed with an equimolar amount of calcium chloride prior to administration. No change in the concentration or distribution of lead in the blood resulted. The rate of excretion of lead in the urine decreased slightly.

Disodium catechol-1,3-disulfonate has been reported to have a favorable effect on the course of experimental lead poisoning by virtue of its chelating action.[13] It was administered to 1 calf at a dose equivalent to 110 mg./kg. Ca EDTA. A tenfold rise in the rate of urinary excretion of lead resulted during the 4-hour period immediately following administration. The rate then fell back to base line for the succeeding 56 hours. A modest increase in the concentration of lead in plasma was noted 15 min. after administration. At one-half hour the concentration had practically returned to base line. In brief, the effect of disodium catechol-1,3-disulfonate was extremely evanescent. No toxic effects were associated with the use of either of these compounds.

Discussion and Summary

The experimental data presented do not shed much light on the numerous individual factors which influence the ability of chelating agents to abstract a heavy metal from the body. There is a strong suggestion, however, that Ca EDTA is not capable of removing more than a respectable fraction of soft tissue lead even when it is introduced into the body in maximally effective single doses. Its failure to remove more than one third of the lead associated with erythrocytes suggests a problem of penetrance. Liver perfusion studies recently reported tend to lend support to this concept.[14] In spite of the distinct possibility that permeability factors may impose serious limitations upon the activity of polyaminoacetic acids, it remains to be demonstrated that there exists a "stability constant plateau" that exhausts the possibility of improving upon detoxification by increasing the stability of the metal-ligand bond.

The data presented concerning the effect of BAL acid and penicillamine on the urinary excretion of lead suggest that dithiol and aminothiol chelating agents should be investigated more extensively. These two tridentate compounds compared rather favorably with the sexadentate polyaminoacetic acids investigated.

Three chelating agents which have been either used clinically in the treatment of lead poisoning or suggested as being potentially useful were found to be either inferior to Ca EDTA or without any noticeable activity. These were BAL, sodium citrate, and disodium catechol-1,3-disulfonate.

A number of other compounds that, for one reason or another, were thought to be worthy of screening had no effect on the excretion of lead in the urine. These included propane-1,3-dithiol, ethane-1,2-dithiol, 2,5-dithiahexane, and 2-aminoethanethiol.

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Part IV. The Industrial Applications of Chelating Agents

CHELATION AND CATALYSIS

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In any discussion of chelating agents or chelation chemistry the subject of catalysis must always be accorded a prominent position. The reason for this is obvious. Chelating agents interact with and modify the properties of metal ions; and it is metal ions—functioning as weak Lewis acids, as efficient electron transfer systems, as perturbing influences on the electron distributions of the molecules around them—that are the actuators in most homogeneous catalysis reactions.

An excellent review of the catalytic effects of chelation and chelate compounds was presented by Martell and Calvin[1] and, more recently, by Chaberek and Martell.[2] The extensive references presented by these authors attest the importance that researchers have attached to the subject. The papers presented elsewhere in this monograph that discuss the role of chelation in enzyme systems and in organic synthesis are examples of data being collected currently on an even larger scale.

For the present discussion it is useful to adopt a classification of catalytic reactions involving chelates from the earlier reference.[2] Under this classification these systems are divided on the basis of the fate of the chelant during the reaction: (1) the metal chelate is not permanently altered during the course of the reaction, and (2) the metal chelate is permanently altered during the course of the reaction.

Examples of reactions of the former class, in which a metal chelate, as such, functions in the role of catalyst are not numerous. That which has received most study is the hydrolysis of fluorophosphorous compounds,[3,4] which was found to be catalyzed by cupric chelates of such materials as ethylenediamine, the simple amino acids, α,α-bipyridyl and o-phenanthroline. The most strongly catalytic were bidentate ligand chelates, and the mechanism would appear to involve interaction between the remaining pair of coordination sites of the copper ion and the substrate molecule to make the phosphorous atom more electrophilic for approach of the water molecule or hydroxyl ion.

A similar dependence upon open adjacent coordination sites in the catalytic chelate is found in work by Wang,[5] who showed that hydrogen peroxide decomposition was catalyzed by the triethylenetetramine chelate of ferric iron. The two oxygen atoms in the peroxide are thought to be drawn to the pair of coordination sites unfilled by the tetradentate ligand, aiding in the breaking of the peroxide linkage.

Reactions illustrative of the second group, those in which the chelate is permanently altered, are more numerous. Perhaps the best known is work done on the autoxidation of ascorbic acid,[6] which serves to show that chelation of trace amounts of added cupric ion by the ascorbic acid serves to "drain" electrons from the ascorbic acid and promote its oxidation. The

well-known lag in the titration of permanganate by oxalate is due to a slow build up of Mn (III), upon which the oxidation is catalyzed through a mechanism thought to involve chelation of the oxalate by Mn (III).[7] The hydrolysis of the condensed polyphosphates is catalyzed by metals,[8,9] the mechanism apparently involving a coordination reaction that draws electrons from the phosphate atom, making it more acidic and prone to attack by water or hydroxyl ion.

The types of reaction discussed under the two groupings above, then, encompass chelate-catalysis reactions. It is important to note that, under the most rigid definition of catalysis, only the first class is representative of true metal-chelate catalyzed reactions. Reactions of the second type are reactions which *proceed* through metal-chelate formation, but perhaps they should be labeled more properly as a special class of metal-catalyzed reaction.

When attention is focused on the commercial applications of chelating agents and the broad areas of chelant usage are enumerated, the area of catalysis is most often included. Chelating agents are said to have use "as catalysts" or "in catalytic processes." However, it is most significant that there is essentially *no* commercial utilization of chelating agents as catalysts under the first classification mentioned above and, further, there is little utility on an industrial scale of catalysis through a chelation mechanism, as outlined under the second heading.

That such practical utilization will develop is quite likely in view of present extensive research efforts in these areas. However, present commercial utility of chelation in the area of catalysis is exclusively in the inhibition of metal-ion catalyzed reactions. The chelant is used to buffer the metal ion concentration and lower the catalytic activity exactly as it is used to buffer the metal ion levels and prevent the formation of hard water soap precipitates.

The most important commercial sequestrants are the condensed phosphates, the polyhydroxy acids and the aminopolycarboxylic acids. Of these, the latter form the most stable metal chelates and, hence, they lower the metal ion concentration to the greatest extent. Since mere traces of metal ion are frequently sufficient to produce extensive catalysis, this higher stability has given the aminopolycarboxylates the greater utility in this application area.

Perhaps the best known use of chelants in the area of inhibition is in the peroxide bleaching of cotton. The usual textile peroxide bleach bath is run under alkaline conditions for optimum bleaching activity. Sodium silicate is added as a stabilizer, but the concentration must be controlled to prevent silicate scale formation. The usual temperature of the baths is near 160° F. and, under these conditions, small concentrations of iron, manganese, and copper bring about extensive decomposition of the peroxide. The use of ethylenediaminetetraacetic acid (EDTA)-based chelants materially reduces such decomposition and permits the baths to be run with less critically high silicate levels.

More recently, chelating agents based on diethylenetriaminepentaacetic acid (DTPA) have been shown to be superior in this application, presumably due to the greater stability of the DTPA chelates. A striking demonstration of the effect of increasing metal chelate stability on the utility of the chelating agent

in preventing decomposition of hydrogen peroxide has recently been shown under mill conditions.[10] A southern textile bleachery had been stabilizing peroxide baths with a chelant based on hydroxyethylethylenediaminetriacetic acid (HEDTA). Under the conditions of the operation the residual peroxide content of the bleached goods had been varying between 10 and 20 per cent. Without making other changes in the system, equimolar amounts of DTPA were substituted for HEDTA. The resultant residual readings increased to the range 50 to 60 per cent. The heavy metal chelate stability constants for DTPA are of the order of 100,000 times those of HEDTA.

Closely related to the metal ion-catalyzed decomposition of hydrogen peroxide is the metal ion-catalyzed autoxidation of unsaturated aliphatic compounds, which are thought to proceed by free radical peroxide chain reactions.[11-13] The role of the metal ion is to decrease the induction period by catalyzing the initial formation of free radical peroxides. Conventional antioxidants function as chain terminators in such systems. The addition of chelating agent to inactivate contaminant metals and inhibit formation of the chain initiators also serves to protect such systems.

Examples of such chelant applications in commercial processes are myriad. Most familiar are the use of EDTA to prevent rancidity and chalking in fatty acid hand soaps,[14] in the stabilization of rubber,[15] and in the refining of vegetable and animal oils.[16] The protection of food products from such metal catalysis represents an interesting application area that is currently being developed on a commercial scale. This topic is the subject of another paper in this monograph.

The single large-scale application for chelating agents that most nearly approaches use as a catalyst is found in the polymerization of styrene-butadiene rubber. The rubber industry has been estimated to utilize between six and fifteen per cent of the annual EDTA production in this country[17] and a significant proportion of this material becomes one of the components of the catalyst system in sulfoxylate polymerization.

Recent investigation[18] on the specific effect of the chelant in this polymerization system has served to indicate that the prime function of the agent is, again, in a metal-buffering capacity. In this work, polymerization reactions were carried out utilizing the typical, cold-rubber, sulfoxylate "recipe" presented in TABLE 1. In this reaction system styrene and butadiene polymerize in an aqueous system, utilizing a fatty acid soap or a resinate soap as an emulsifier.

The activator system consists of ferrous iron, which interacts with an organic hydroperoxide to produce peroxide free radicals that, in turn, produce free radical monomers. The self-sustaining polymerization then proceeds, with the polymer growing in the micelles. Sodium formaldehyde sulfoxylate is present to reduce ferric iron to the ferrous state, and the activation cycle then is repeated. In this system relatively minor amounts of iron are utilized and the presence of contaminant iron in the product is minimized.

A chelating agent in the system is essential, but uncertainty has existed over whether the true activating species is the free metal or the metal chelate, that is, whether the chelant is functioning as a true catalyst or simply as a buffering agent. For this work a series of available aminopolycarboxylic acid-based

TABLE 1*

COMPOSITION OF POLYMERIZATION SYSTEM USED IN STUDYING EFFECT OF CHELANT

Recipe	
Butadiene	70 parts
Styrene	30 parts
Deionized water	180 parts
Diisopropyl benzene hydroperoxide	0.10 parts
Sodium formaldehyde sulfoxylate dihydrate	0.07 parts
Potassium palmitate	4.50 parts
Tertiary dodecyl mercaptan	0.30 parts
Potassium chloride	0.30 parts
Ferrous sulfate heptahydrate	0.02 parts
Chelating agent in various molar ratios to iron	
Temperature	5° C.
*p*H	10.5–10.6

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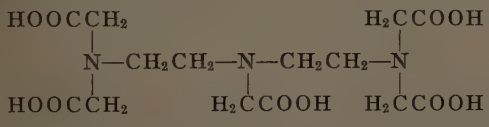
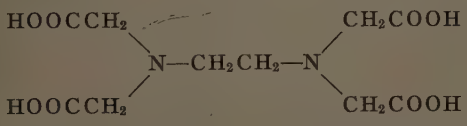
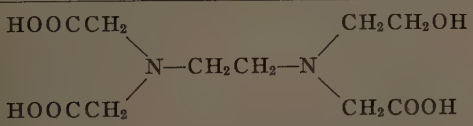
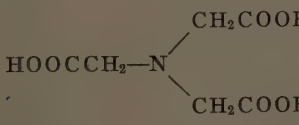
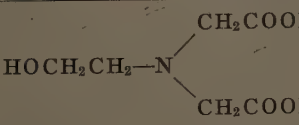
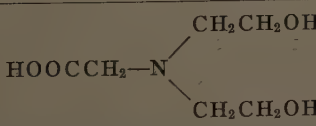
TABLE 2*

STRUCTURAL FORMULAS AND IRON CHELATE STABILITY CONSTANTS FOR CHELANTS USED IN POLYMERIZATION STUDY

Chelating agent used		Log stability constants	
		Fe^{+++}	Fe^{++}
	DTPA	28.6	16.5
	EDTA	25.1	14.3
	HEDTA	19.6	11.6
	NTA	15.9 24.3†	8.8 —
	HEIDA	11.6 —	6.8 10.0†
	DHEG	— —	4.3 7.3†

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† 2:1 Chelate.

chelants were used. As may be seen from TABLE 2, the series included chelants which varied in their iron-chelating ability.

It was found, in the representative system tested, that only chelants based on EDTA supported and sustained the reaction (FIGURE 1), giving nearly linear per cent conversion versus time plots, reaching 60 per cent conversion in six to eight hours. Thus chelants that were more effective in tying up iron than EDTA (for example, DTPA) and those that were less effective (NTA, DHEG)

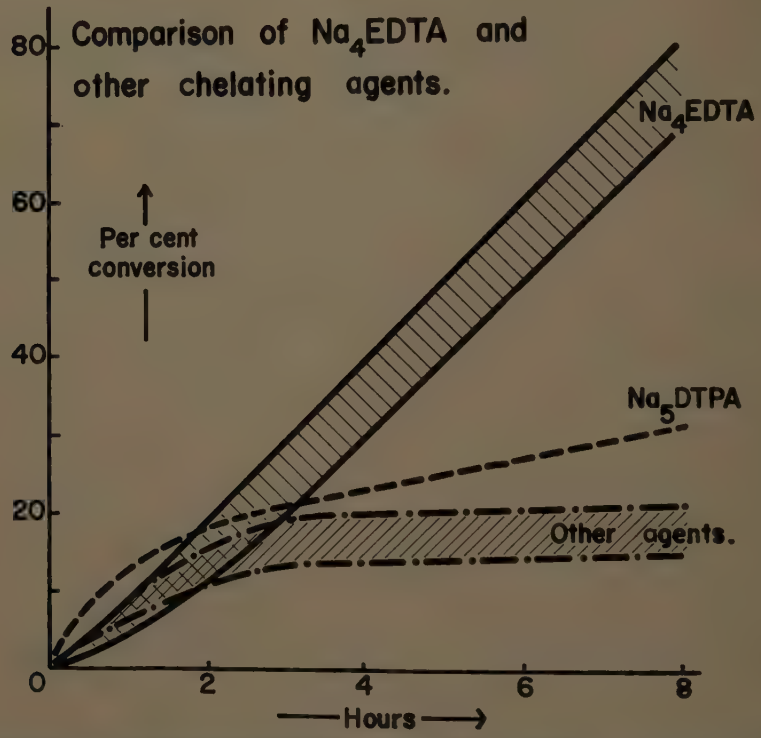

FIGURE 1. Conversion curves showing unique utility of EDTA in polymerization system. Reproduced, with permission, from *Industrial and Engineering Chemistry* (in press). Copyright 1960 by The American Chemical Society.

were equally ineffective in the activation system. For the investigation EDTA, DTPA, and HEDTA were used in amounts equimolar to the iron, while NTA, HEIDA, and DHEG were used in 2:1 molar ratio to iron.

The results of another series of experiments, in which the ratio of EDTA to iron was changed, are presented in FIGURE 2. It was shown that radically reducing the chelant-to-iron ratio produced only minor variations in the conversion curves, a ratio of 0.6 being, within experimental uncertainty, as effective as a ratio of 1.0.

Based on the above studies it is possible to draw some conclusions about the effect of the chelant in the system. Because at *p*H 10.5 the ferric chelates of all these agents are extensively hydrolyzed, the ferric ion level must be main-

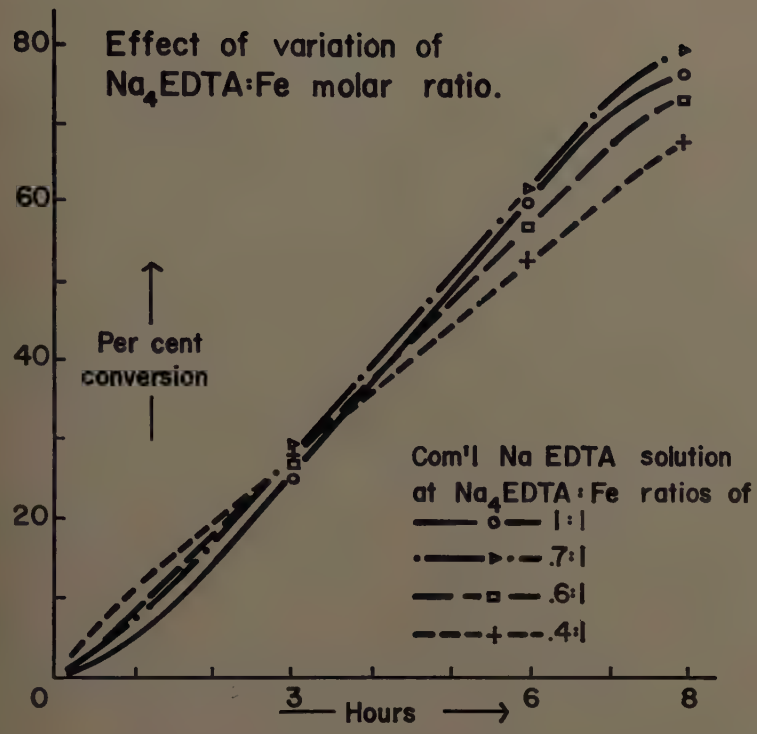

FIGURE 2. Conversion curves showing relatively small effect on lowering chelant-to-iron ratio. Reproduced, with permission, from *Industrial and Engineering Chemistry* (in press). Copyright 1960 by The American Chemical Society.

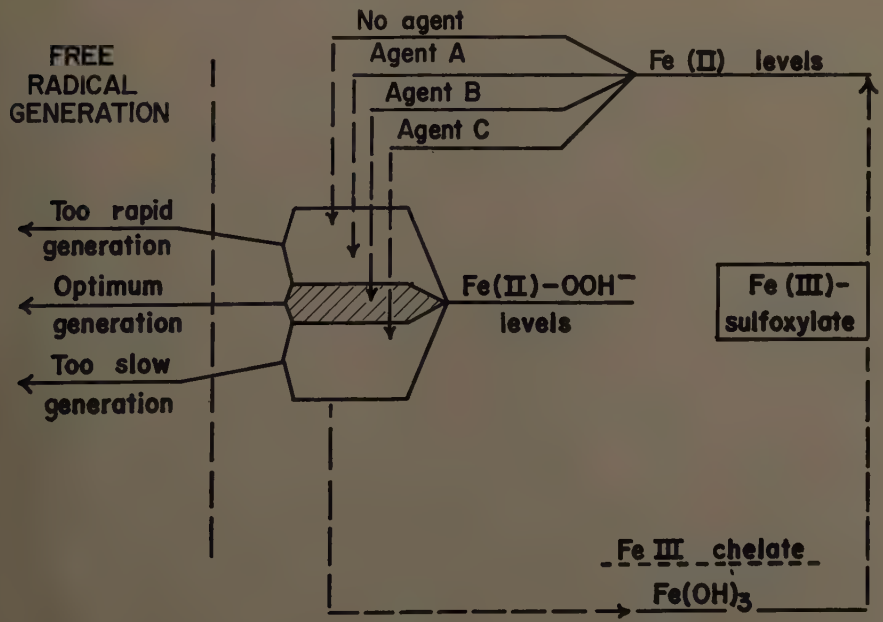

FIGURE 3. In activation system, chelant B maintains free Fe (II) level for optimum peroxy-free radical generation. Fe (III) level is maintained by *p*H of system or by reduction by sulfoxylate.

tained either by the *p*H of the system or at a steady state by reduction by sulfoxylate. The chelating agent in the system is essentially freed to control ferrous ion, and it is possible to use lower than stoichiometric chelant-to-iron concentrations.

For all the chelating agents used, and for a given ferrous iron concentration in the system, the concentration of chelated ferrous ion will be essentially the same. The factor of differing ferrous chelate stabilities will have its greatest effect on the concentration of free ferrous ion that is maintained. Thus it is reasonable to conclude that the exclusive utility of EDTA in the system is due to the fact that the free ferrous ion concentration is maintained at a level for optimum formation rate of free radical peroxides (FIGURE 3). Were the reaction catalyzed by the ferrous chelates, the differing agents would be expected to produce varying degrees of activity in the system.

Thus the utility of the chelant appears to be based upon its metal-buffering capacity, and the first commercial scale utility of chelant in a true catalytic capacity is still to be developed.

The above remarks have concerned themselves exclusively with homogeneous catalysis. In conclusion, it is interesting to speculate upon the potential utility of chelation mechanisms in heterogeneous catalysis systems. Several instances could be mentioned in which chelants modify the activity of surfaces, and such perturbation might produce useful modification of catalytic activity of the surface.

It seems certain that the research emphasis currently being placed on chelation and catalysis will result in most interesting developments on the industrial scale in the next few years.

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PROPERTIES AND APPLICATIONS OF METAL ACETYLACETONATES

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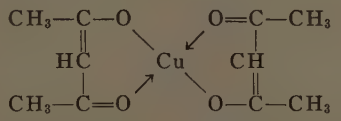
Acetylacetone, whose enol form is a weak acid[1] with dissociation constant $K_a = 1.12 \times 10^{-9}$, readily forms metal salts. The acetylacetonates of virtually all the metals in the periodic table have been prepared and reported in the literature. These compounds are characterized by unusual thermal stability, volatility, and solubility in organic liquids; these properties have given rise to many proposed uses for the compounds.

Some common physical properties of a few representative metal acetylacetonates are collected in TABLE 1. The compounds are not very soluble in water and their dissociation into ions is slight.[2] Thus copper acetylacetonate is stable to boiling in amyl alcohol,[3] even on addition of anhydrous or concentrated aqueous K_2CO_3, although it is immediately destroyed by KOH. These results depend upon sequestration of the metal ion by the acetylacetone ligands through coordination:



The formation constants for the metal chelate ions given in TABLE 1, that is, the reciprocals of the dissociation constants

$$K_1 : M^{++} + Ch^- \leftrightarrows MCh^+$$

$$K_2 : MCh^+ + Ch^- \leftrightarrows MCh_2$$

indicate the relative stabilities of the metal derivatives. They increase markedly[2] as the solvent becomes less polar. This accounts for the fact that solubilities of metal acetylacetonate compounds in water are usually greatly enhanced by the addition of a hydrophilic organic solvent such as dioxane.

The volatility of the metal derivatives, also indicated in TABLE 1, is quite remarkable and is another illustration of the bond strengths in these compounds. The chelated structure given should be completely nonpolar, yet the degree of polarization found by dielectric constant and dipole moment measurements, both in the liquid and gaseous states, is higher than expected, by a factor of 50 per cent,[4] and indicates some type of internuclear polarization within the molecule.

The stability of these chelates and their resistance to hydrolysis have been put to practical use. Methods for extracting metals from solutions and from ores have been developed, based on the formation of these complex compounds. Aqueous solutions containing copper and zinc have been extracted[5] with acetylacetone at *p*H 2.1; at this acidity copper acetylacetonate forms preferentially and transfers to the organic layer, with the partition so sharp that an analytic separation could be based on the method. Acetylacetone extractions have been

TABLE 1
PROPERTIES OF REPRESENTATIVE METAL ACETYLACETONATE COMPOUNDS

Metal	Color	Cryst.	M.P. (°C.)	B.P. (°C.)	Sublimation	Solubility			Formation constants		Density
						Water	Benzene (gm./100 cc.)	Ethanol	Log K_1	Log K_2	
Na^{+}	White	Hex. plates	218	d.		Sol.	Insol.	Sol.	4.4	—	d_4^{20} 1.213
Be^{++}	White	Monoclinic	108	270	Readily	Sol. hot	30	Sol.	7.8	6.7	d_4^{20} 1.108
Zn^{++}	White		124 d.	d.	Partial 124° C.	d. to basic salt at 100° C.	Insol.	Sol.	5.0	3.8	
Ni^{++}	Green	Dihydrate	228	230 11 mm.	220° C.	Insol.		106 gm./l.	5.9	4.5	
Al^{+++}	White	Monoclinic	193	314 d.	140° C. 10 mm.	<3.3%	41.6	V. sol.	8.8	8.0	d_4^{20} 1.204
Fe^{+++}	Red		184	d.		<3.5%	57.7	Sol.	9.8	9.0	1.33
La^{+++}	White		142	d.		<4.4%	0.05	Sol.	5.1	3.8	1.542
Zr^{++++}	White		193	d.	Slight at 82° C., 0.001 mm.	Insol.	7.0	200 gm./l.			d_4^{25} 1.415

used similarly in ferrous[6] and vanadium[7] analyses, and the extraction of both thorium[8] and zinc[9] ions from their solutions has been studied.

In my laboratory, manganese ores have been extracted with acetylacetone to separate the manganese values. A low-grade pyrolusite ore, 100 gm., containing 27 per cent manganese, was ground to 200 by D fineness and leached with acetylacetone. The effects of time, temperature, and ore-to-solvent ratio

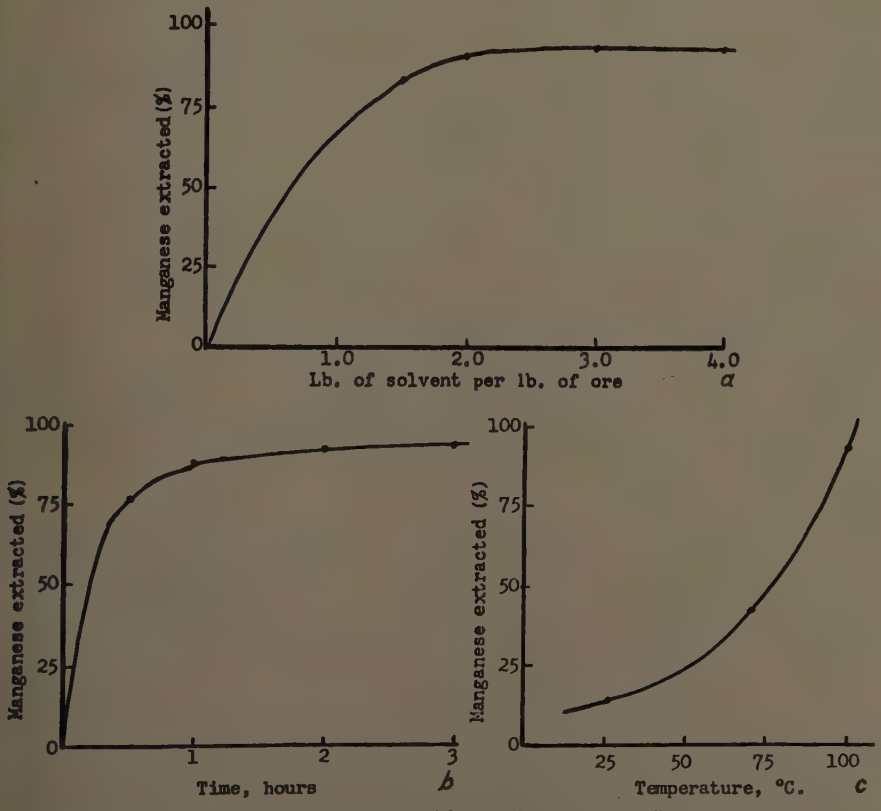


FIGURE 1. Extraction of manganese ore with acetylacetone. *A*, effect of ore:solvent ratio (t = 129° C., time = 2 hours). *B*, effect of time (t = 129° C., ore:solvent = 1:4). *C*, effect of temperature (2 hours; ore:solvent = 1:4).

on the extraction are shown in the adjoining FIGURE 1; 90 per cent of the manganese is extracted from the ore by leaching it with 3 times its weight of acetylacetone for 1 hour at 100° C.

Uranium also may be extracted from ore by direct leaching with acetylacetone. Results obtained in the working of 200-gm. samples of 270 by D carnotite ore containing 0.3 per cent of U_3O_8 are shown in FIGURE 2. After 6 extractions with solvent at 129° C. more than 95 per cent of the uranium was leached from the ore.

These procedures for recovering metal values from ores are being considered for industrial use. The economics of the processes depend upon recovering the

acetylacetone remaining on the gangue material, for instance, by leaching with acetone and subsequent fractionation of the mixed solvents. After the metal acetylacetonate compounds have been isolated or concentrated they can be decomposed with a strong acid and the acetylacetone distilled off for re-use.

Applications

The many proposed industrial uses for the metal acetylacetonate compounds depend on one or another of the unusual properties these materials possess. Since the compounds are soluble in organic solvents, they provide an easy means of carrying metal ions into important classes of industrial materials such as rubber, plastics, gasoline, lubricating oils, paints, and enamels. Here the metals can exert their characteristic effects.

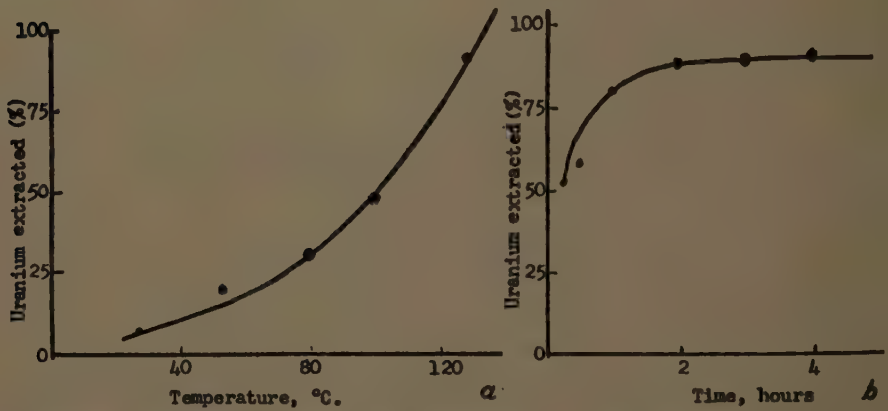


FIGURE 2. Extraction of uranium from carnotite ore. *A*, effect of temperature (3 hours; ore:solvent = 1:2). *B*, effect of time (129° C.; ore:solvent = 1:2).

Catalysis is one of these effects. Metal acetylacetonates have been recommended as catalysts for oxidation of hydrocarbons,[10] for polymerization of unsaturated hydrocarbons[11] and of silicone monomers,[12] and for acidolysis reactions.[13] Their use as drying agents in linseed oil[14] and as accelerators for the vulcanization of rubber[15] also illustrates their catalytic properties. Their use as ignition and combustion catalysts in gasoline and motor fuels has been investigated with particular thoroughness;[16-25] fractional percentages of the additives impart antiknock value to the fuel and improve engine cleanness, it is claimed. Used in lubricating and fuel oils, they catalyze the combustion,[26] prevent sludge and hard carbon deposits from forming,[27-28] and act as soot-removal agents.[29] The utility of the metal acetylacetonates as combustion promoters in jet fuels[30-31] is particularly noteworthy. Chromic acetylacetonate increases markedly the power output of nitromethane when used in a rocket motor. It also decreases the tendency of nitromethane to detonate under shock.[32] It is claimed that the improvement of antiknock value of hydrocarbon fuels comes about not so much because of prevention of formation of formaldehyde and peroxidic materials as by breaking reaction chains[16] once they

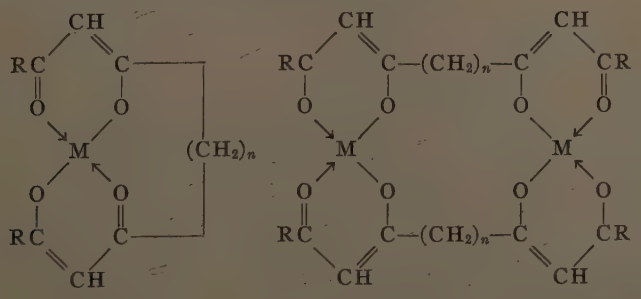
get started. On the other hand, evidence is available that the metal derivative does not improve combustion directly but rather by being burned to metal-containing deposits which, on the walls of the combustion chamber, provide the more efficient combustion.[17]

The volatility and easy decomposability of the metal acetylacetonates have been put to use in several processes for metal plating. Pawlyk[33] passes carbon dioxide at 400° F. over solid copper acetylacetonate in order to pick up vapors of the latter; the mixed vapors are impinged onto steel or other material to be plated, which is held at such temperature (650° F.) as to decompose the chelate compound and deposit the copper as a smooth continuous coating. Methods of plating other metals than copper by this procedure remain to be developed. Marboe,[34] in a similar process, volatilizes various metal acetylacetonates at 350 to 400° F. under vacuum and deposits metal coatings on glass. If air or oxygen is bled into the vapors prior to impingement on the material being plated, metal oxides are deposited rather than metals, and this behavior is utilized[35] in coating dielectrics and heat-insulating materials.

In the plastics industry, besides their use as polymerization catalysts, as noted above, metal acetylacetonates have been proposed as stabilizers for acrylonitrile polymers,[36] as regulators for methacrylate polymerization,[37] and as curing agents for epoxy resins.[38-39] They promote the adhesion of foamed urethane plastics to covering layers of all kinds in the making of curtain-wall materials.[40] Titanium acetylacetonate was investigated as a cross-linking agent in cellulosic lacquers[41] for improving the strength of the plastic.

Intermediates

In 1953 Wilkins and Wittbecker[41] melted beryllium acetylacetonate with terephthaloyldiacetone and, after distilling out some acetylacetone, obtained a solid resinous polymer melting above 285° C. Polymers were generally obtained by melting mixtures of metal acetylacetonates and bis-β-diketones or by reacting solutions of the bis-β-diketones with metal carbonates or acetates. Molecular weights are not reported, but the products could be molded, spun, or cast. This work is interesting in that it points to the possibilities of achieving thermally stable, semi-inorganic polymers based on metal acetylacetonate structures. A multifunctional substituted acetylacetone of the type R—CO—CH—CO—$(CH_2)_n$—CO—CH—CO—R can react with a bivalent metal ion in 1:1 ratio and form monomer, dimer, or polymer:



Monomer (A) Dimer (B)

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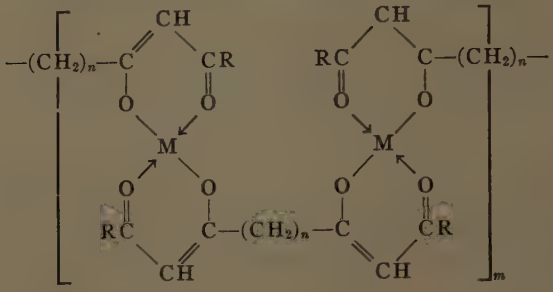

Polymer (C)

The monomer is less likely to form if n is small (less than 4 or 5), and dimers of the type shown have been demonstrated only for copper-di-imine complexes. The stabilities of metal acetylacetonate polymers (C) have been shown[42] to parallel those of the simple metal acetylacetone complexes, that is, for divalent metal ions, Cu > Be > UO_2 > Ni > Co > Zn > Mn > Mg. Such polymers are easily formed (a) by mixing a metal acetylacetonate with a tetraketone in aqueous or organic solution, with or without the addition of alkali to effect precipitation, (b) by melting the metal acetylacetonate compound with a bis-β-diketone and boiling out by-product acetylacetone or acetic acid to the desired product viscosity, or (c) by refluxing a bis-β-diketone over a metal oxide, carbonate, or basic acetate, with elimination of water, carbon dioxide, or acetic acid. By these methods one achieves polymers (C) with m as much as 10 or 12; the problem of getting very high molecular weight polymers of this type is still unsolved.

Apart from polymers, metal acetylacetonates have served as intermediates for the synthesis of other substances. Cobalt acetylacetonate[43] has been the starting point for making cobalticinium bromide, $Co(C_5H_5)_2$ Br, and indium acetylacetonate,[18] for making various organic compounds. A variety of miscellaneous applications for metallic acetylacetonates has been reported: as insecticides,[44] as addition agents in electroplating baths,[45] as rust solvents,[46] and as pore extenders.[47] Now that various metal acetylacetonates are becoming commercially available much wider application of these interesting compounds is to be anticipated.

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MAINTAINING FOOD QUALITY WITH CHELATING AGENTS

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"The food scientists of this nation are engaged in improving our food supply by making it more acceptable—not less acceptable; in making it safer—not less safe. Millions have been spent in testing additives—more millions will be spent. Millions have been spent in improving food processes, to produce cleaner food materials and better to handle raw materials once they are harvested. The improvements in sanitary practices alone costing millions of dollars have raised the quality level of our food supply above that of any nation. Routine sampling and analyzing of food in process to assure its high level of quality is painstakingly done with every tool at our disposal to assure the consumer that he gets only the finest that can be produced.

"This program will require the complete cooperation and understanding of all—industry, government, and academic. We in industry will continue to perform our functions to give to the nation a safer and better food supply through the application of improved technology in its broadest concept."

I. J. Hutchings
President-elect, I.F.T.*

When we talk about food and the food industry we are talking about a most dynamic and rapidly changing industry. To illustrate, about two thirds of the sales of grocery stores (today) are products that are entirely new or have been basically improved in the last 10 years. Of that two thirds, about 50 per cent are entirely new items and the other 50 per cent are basically improved, such as the "instant" products coffee, tea, and oatmeal. A large food chain has indicated that its buyers are presented with 24 new products every day, or 6000 new products a year, on the supermarket shelves. Although many of these products are duplications, their sheer number does illustrate the increasingly stepped up efforts of the food industry to provide the housewife with quality convenience products having good recipe tolerance and stability.

The tremendous growth of convenience foods means that more actual food preparation is being done by the food processor. Today the housewife spends about 1½ hours preparing three meals, whereas a few years ago she spent about five hours. With more preparation by the processor, there is a trend toward greater distances between the food production plant and the consumer. In addition there is an increasing demand from the public for a wider variety of food for developing menus.

Among the food additives that will play an important role in helping the food processor prepare high-quality foods are chelating agents. The term chelating agent is a generic name describing a function. Many different compounds can perform this function. For instance, the porphyrin structure, with its four coordinate bonds resulting in six very stable ring structures, is a very effective and strong natural chelating agent. Amino acids, polyphosphates, and organic acids are also chelators, but have less effectiveness because they

* Talk by I. J. Hutchings to joint session of the Food Law Institute with the Food and Drug Administration, Washington, D. C., November 17–18, 1959. Reprinted by permission of the author.

form weaker complexes. One of the most effective chelating agents that can be produced economically for food systems is the disodium salt of the calcium chelate of ethylenediamine tetraacetic acid (Ca EDTA).

Why is a chelating agent so important in maintaining food quality? We have long known the importance of trace elements, such as iron and copper, in the human diet. Only relatively recently, however, have we recognized the undesirable effects of free metal ions in food products. Among such effects are loss of eye appeal, flavor, texture, nutritional value, and over-all acceptability. Addition of a strong chelating agent serves to inactivate these metal ions, yet does not destroy the nutritional value of these trace elements.

From the literature and the present industrial uses of chelating agents, it can be postulated that the undesirable effects of active heavy metal ions such as iron and copper can be produced in at least two ways. First, heavy metal ions are catalysts in the oxidation of double bonds and can affect the stability of unsaturated fatty acids and such vitamins as ascorbic acid. Second, they can react with higher molecular weight components in foods to yield insoluble complexes. Thus iron reacts with tannins to product iron tannates, which cause undesirable blackening in such products as cauliflower and asparagus.

Stability of Fats and Vegetable Oils

Fats and oils are subject to two forms of instability—hydrolysis and oxidative degradation. Early stages of oxidation and hydrolysis are commonly associated with reversion, which can be detected as off-flavors in such foods as milk or in oils such as soybean oil. The more advanced stages of oxidation result in rancidity, which is readily detected from both flavor and aroma.

Over the years a number of practices have been evolved that help to minimize degradation of fats and oils:

(1) Hydrogenating unsaturates to saturates. This procedure reduces the ease of oxidative attack, since degradation increases markedly with the number of double bonds. Advantage is taken of hydrogenation in preparing such products as shortening but, even so, traces of metallic hydrogenation catalysts can cause stability problems. However, with the increased usage of unsaturated fatty acids some other technique must be used to reduce instability.

(2) Maintaining low storage temperatures.

(3) Using opaque or colored glass to prevent initiation of oxidation by light.

(4) Preventing the product from contacting or absorbing excess air during processing, as well as attempting to maintain the natural antioxidants present.

(5) Minimizing contamination with various pro-oxidant metals such as copper and iron.

However, even when all these practices are observed and antioxidants added, degradation still occurs and can be as important in foods with low fat content as in those with high fat content.

Early studies of trace metal effects evaluated such compounds as citric acid, sorbitol, tartaric acid,[1] esters of citric acid,[2] and ascorbic acid.[3] The effects of citric and ascorbic acid with the antioxidant lauryl gallate in a lard system[3] are shown in TABLE 1. The stability index is obtained by division of the stability time for the product with additives present by the stability time for the control.

Any value greater than 1 represents stability improvement. First, the detrimental effects of traces of copper and iron are quite evident from the reduced stability of the control lard with no additives present. The addition of each of the three compounds separately gave improvement in some cases, but they were not generally effective. The greatest improvement in stability resulted when either ascorbic or citric acid was added with the lauryl gallate. The citric acid combination was superior, because it gave control against iron as well as copper.

Aqueous fat systems probably are more difficult to stabilize than pure fat systems, since many antioxidants have appreciable water solubility and in

TABLE 1
LARD STABILITY INDEX (BASED ON AOM STABILITY TESTS)[3]

Lard and additives	No added metal	Cu, 0.2 ppm	Fe, 2.0 ppm
Lard + 0.01% lauryl gallate	7.30	8.13	1.4
Lard + 0.01% ascorbic acid	1.43	12.50	1.0
Lard + 0.01% citric acid	1.0	2.75	9.0
Lard + 0.01% lauryl gallate + 0.01% ascorbic acid	9.0	75.0	1.4
Lard + 0.01% lauryl gallate + 0.01% citric acid	8.0	41.3	80.0
Control lard	7 hr.	0.8 hr.	0.5 hr.

TABLE 2
STABILITY INDEX FOR LARD IN CONTACT WITH AN AQUEOUS PHASE[4]

Additives (0.005% tocopherol added to all samples)	Borate buffer, *p*H 7	Phosphate buffer, *p*H 5.6
Lard + 0.1% ascorbic acid	0.08	<0.16
Lard + 0.05% Versene	5.05	1.17
Lard + 0.1% ascorbic acid + 0.05% Versene	>11	30
Buffer control	9 days	0.5 day

emulsions do not have the same protective power as if they were retained completely in the fat phase. In a study of an artificial lard-water system[4] the use of (Na_4 EDTA) with ascorbic acid reportedly gave effective control of trace metal ions and markedly improved the lard's stability. This effectiveness was shown to be maintained under varying conditions of temperature, *p*H, and addition of tocopherol and hemoglobin. TABLE 2 shows the stability index for such a series of tests and, again, illustrates the higher degree of stability obtained when a chelating agent is used in combination with an antioxidant. In this test one other interesting fact was noted: the addition of equimolar or higher quantities of calcium chloride had only a very slight effect on the action of Na_4 EDTA.

A 1959 patent[5] demonstrates the unique ability of EDTA salts and calcium chelates to maintain the quality of emulsified salad dressings. Specific examples of French dressing and mayonnaise were cited. At the end of storage tests

at 72° and 98° F., products protected by 0.001 per cent EDTA showed no development of the characteristically musty mustard flavor, no deterioriation in the vegetable oil, and no appreciable color change. Retention of the spice flavor was also noted.

Although EDTA is not soluble in fats and oils, considerable stability can be incorporated by the addition of EDTA to the oil during the cooling step of deodorization. The use of 0.01 per cent EDTA acid in such a manner with soybean oil markedly reduced titratable peroxides when stability was tested by the active oxygen method.[6]

While the use of EDTA is effective, one question arises: will fat-soluble chelating agents giving greater improvement in stability ultimately be found? Recent research along this line has disclosed two new fat-soluble metal deactivators.[7] These compounds are the monooctadecyl esters of either (carboxy-methylmercapto)succinic acid or thiodisuccinic acid.

Stability of Ascorbic Acid

As mentioned above, EDTA provides vitamins such as ascorbic acid with improved antioxidant properties. Ascorbic acid occurs naturally in many foods, particularly citrus juices, and supplemental quantities also are frequently added.

In tomato juice[8] the flavor quality of stored juice was noticeably poor when about one-half the ascorbic acid content was lost and the amino nitrogen content was 10 per cent of its original value. This loss of ascorbic acid by oxidation is catalyzed by traces of metal ions, in particular, copper.

At normal storage temperatures, canned orange juice has a short shelf life because of the rapid development of off-flavors and loss of ascorbic acid. Although considerable improvement can be obtained by keeping the dissolved oxygen content below 0.8 per cent,[9] ascorbic acid loss still occurs. This is ascribed to copper and, possibly, iron, since pasteurization would destroy any enzymatic degradation processes.

The addition of small amounts of Na_2 EDTA to acidified solutions of ascorbic acid protects the vitamin against copper-catalyzed oxidation.[10] Quantities of Na_2 EDTA as low as 0.2 mmoles were found effective. With passion fruit juice[11] 20 to 30 ppm (juice basis) Na_2 EDTA gave more than 50 per cent inhibition of ascorbic acid loss. Higher quantities in excess of the metal ion content gave 90 to 100 per cent inhibition of oxidation in boiled juice and 80 to 90 per cent inhibition in unboiled juice. In a study of grapefruit juice 1×10^{-4} *M* copper ion caused 84 per cent loss of the ascorbic acid in two days at a temperature of 2° C.* When Ca EDTA was added at 1×10^{-3} *M* concentration, the ascorbic acid loss was limited to about 15 per cent over a period of five days.

Stability of Other Foods

The usefulness of chelating agents such as EDTA in improving food product quality is not limited to preventing metal-catalyzed oxidation. Metal-protein reactions likewise are a common source of quality problems. Copper and protein react to give a cloudy appearance to apple juice[12] or to cause the common

* P. A. Wolf, The Dow Chemical Company, Midland, Mich.; unpublished data.

cassé problem in wines. The ability of EDTA (Na salts or calcium chelate forms) to correct the latter problem has been extensively studied,[13,14] and EDTA very probably could correct the problem in apple juice. Metal-protein reactions appear to be responsible for the "wildness" problem with beer. Two patents have been issued that point out the ability of EDTA to minimize or prevent this problem.[15,16] The so-called roughness or chalkiness of dry milk is due primarily to insoluble calcium-protein aggregates.[17] Two means of correcting this with the use of chelating agents or ion exchange treatment have been suggested.

Polyphosphates likewise have been used by the food industry and in applications where EDTA might well be evaluated. In cooked meats the flavor and odor can rapidly change during storage, and tests have established a correlation between stability and peroxide values as determined by 2-thiobarbituric acid analysis (TBA analysis). At a level of 0.5 per cent, tripolyphosphate, hexametaphosphate, or pyrophosphate added to pork maintained a constant low TBA result.[18] When they were used in combination with ascorbic acid, the low TBA figures were maintained for much longer periods of time.

Pregelatinized starches find widespread use in many food products, such as icings, sauces, custards, and many "instant" foods that require that the starch be dispersible in cold or warm liquids. Cereal starches are the most desirable here, but they can give off-flavors and rancid flavors very quickly. The off-flavor can be masked by sugar, flavorings, milk, and other additions. Several chelating agents including EDTA have been found effective in preventing both the development of rancidity as well as the other off-flavors.[19]

Vegetable salads are very difficult to stabilize. Usually they require constant refrigeration and even then have a maximal shelf life of only about two weeks. Very special handling and refrigeration can prevent spoilage beyond this time, but not deterioration of flavor. Considerable improvement, however, may be obtained by the use of EDTA and an edible fungistat.[21] The preferable concentration of EDTA is 0.004 to 0.2 per cent; it can be added to the vegetable component or to the dressing component or to both. Likewise, the fungistat (sorbic acid, crotonic acid, or other) is added preferably in concentrations of 0.05 to 0.15 per cent to either phase or both phases. The fungistat is needed for giving optimal shelf life when the salad contains 60 per cent or more of the vegetable component, but is optional, when less than 60 per cent. When this combination was used, the previous two-week stability was increased to more than a 16-week stability.

Most of the recent work with chelating agent additives has involved EDTA. In addition, there is an increasing use of the preformed calcium chelate of EDTA, since for all metals except the alkaline earth metals Ca EDTA is as effective as the sodium forms of EDTA. Ca EDTA also has the desirable feature of greater safety in use.

Apart from its effectiveness in controlling a problem, a food additive, to be acceptable for use, must display other important characteristics. The additive should not impart any foreign odor, flavor, or color to the food product, nor affect its nutritive value, nor conceal inferiority or faulty processing. It should be effective given in small quantities, economical to use, easily applied during processing, and specific in its actions.

Since Ca EDTA and Na_2 EDTA possess all these properties, it is understandable that food processors are becoming more and more interested in their possibilities.

Conclusion

How will the food processor benefit from chelating agents?

The most direct benefit will be the added insurance that his product reaches the consumer in a state most nearly like the state in which it was prepared. Furthermore, the use of chelating agents will afford him increased freedom to develop new products and recipes.

How will the consumer benefit from chelating agents?

The consumer looks primarily for four qualities in the product purchased:[20] (1) palatability, (2) convenience, (3) economy, and (4) nutrition. Palatability may be improved by enhancing color, flavor, texture, and over-all acceptability. More convenience foods will be available owing to the increased freedom the food processor will have in preparing his products. The economics of various products can be improved, because spoilage losses can be reduced. Full nutritional value can be received from such essential dietary substances as ascorbic acid and linoleic acid.

Summary

This paper outlines the wide variety of problems caused by trace metal ions, and the versatility of small concentrations of EDTA in alleviating or eliminating them completely.

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